STEROLS IN GERMINATING SEEDS AND DEVELOPING
SEEDLINGS OF LONGLEAF PINE, Pinus palustris Mill.

By

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A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF
THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA
1976

ACKNOWLEDGMENTS

I wish to express my sincere appreciation and gratitude to Dr. R. E. Goddard, Chairman of the Committee, for his advice and assistance throughout the graduate program, and his valuable help in preparation of this manuscript. My gratitude, in particular, is expressed to Dr. R. H. Biggs, who served as Co-Chairman of the Committee, for his moral support, his foresight in initiating the experiments, his excellent guidance and help throughout the course of the research, and his patient assistance in preparation of the whole manuscript. My special thanks also is given to Dr. L. A. Garrard, Dr. T. E. Humphreys, Dr. C. M. Kaufman, and Dr. R. M. Roberts, members of the Dissertation Committee, for their valuable criticism and aid in correction and improvement of the manuscript.

I am deeply indebted to Dr. J. L. Gray, Director of the School of Forest Resources and Conservation, for his recommendation for my admission to the Graduate School, and for his support for completion of my graduate program.

My deep acknowledgment is made to the late Professor R. G. Stanley for his excellent supervisorship in guiding my professional development, his valuable suggestions and stimulations of my interest in this problem, and his patient guidance during my early stages of research.

Numerous others extended invaluable assistance at crucial periods of this study, in ways large and small. Dr. R. W. King, Dr. M. H. Gaskins, Dr. C. A. Hollis III, Mr. J. E. Smith, Mrs. S. G. Mesa,

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March 1976

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The presence and possible functions of sterols in germinating

embryos and young seedlings of longleaf pine (Pinus palustris Mill.)

were studied at different periods after germination. Sterol analyses

were performed by gas-liquid chromatography (GLC) and verified by

combination of GLC-mass spectrometry. Campesterol and β-sitosterol

were two major sterols which accounted for most of the sterol composi-

tion while stigmasterol was present only in very small amounts. No

cholesterol was revealed by GLC-mass spectrometry although there was a

minor peak appearing on the sterol gas-liquid chromatograms with a re-

tention time close to that of authentic cholesterol.

By fractionation, three different forms of sterols were obtained:

steryl esters, steryl glycosides, and free sterols. The sterols were

mainly found in the esterified fraction, while steryl glycosides and

free sterols only made up a small portion of the total sterol value.

The total sterol content in general increased during seedling

development, and this increase reflected mainly a change in steryl

esters. The low levels of both free and glycosidic sterols remained nearly unchanged throughout the experimental germination period. These results were in agreement with the suggestion that sterols increase during germination as a consequence of increased membrane and organelle production.

Cycloheximide, abscisic acid (ABA), and the animal steroid inhibitor SKF 7997-A_3 had significant effects on the biosynthesis of sterols, the content of chlorophyll, and the concentration of reducing sugars. The activity of isocitrate lyase, a key enzyme necessary for converting fatty acids to carbohydrates in germinating fatty seeds, was sharply reduced when decoated pine seeds were treated with SKF 7997-A_3 prior to germination. This depression in enzymatic activity was not repaired by administration of sterols to the steroid inhibitor-treated seeds.

Results indicate the involvement of sterols, either directly or indirectly, with the metabolism of germinating seeds and the growth and development of young seedlings of longleaf pine.

INTRODUCTION

Although steroids are known to play very important roles in animal
life, their significance in higher plants is still obscure [111, 118].
Considerable information has been gathered on the biochemistry and
physiology of steroids in animals, but investigations of the function
and metabolism of plant steroids have only recently begun [113, 117,
120, 154].

In recent years, modern analytical methods have been used to iden-
tify and quantify steroids from plants [153]. These analyses have led
to the discovery that most steroids found in animals are also present
in plants [114, 154]. The major sterols found in germinating seeds of
different species have been reported as β-sitosterol, stigmasterol,
campesterol, and cholesterol [111]. The latter was once thought to
occur only in higher animals.

Very little is known about the exact role of steroids in plants
[111]. Some plant steroids have a very profound effect on animals, and
it is unlikely that they have no effect at all on the plants in which
they occur [111, 118].

It has been suggested that steroids may function similarly in
plants and animals [117, 120]. Various exogenously applied steroids
have been reported to affect growth and development of shoot and root
[14, 16, 123], influence sex expression in dioecious plants [172],
accelerate water absorption and germination [15, 19, 119, 120, 185],
and influence chlorophyll synthesis [17]. Inhibitors of animal steroid
biosynthesis, when applied, also inhibit flowering in certain plants

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[41, 89, 206]. An inhibition of sterol biosynthesis and a retardation

of stem growth were observed in tobacco seedlings when they were treated

with three familiar plant-growth retardants: 2'-isopropyl-4'-

(trimethylammonium chloride)-5'-methylphenylpiperidine carboxylate

(Amo 1618), β-chloroethyltrimethylammonium chloride (CCC), and tributyl-

2,4-dichlorobenzyl-phosphonium chloride (Phosfon D) [67]. These obser-

vations indicate the possible involvement of sterols, either directly

or indirectly, in plant growth and development. These metabolites are

widely distributed in the plant kingdom and should no longer be con-

sidered "waste products" [210].

One of the best systems in plants for steroid studies is that of

developing and germinating seeds [111]. However, studies of sterols,

the major group of plant steroids, have usually dealt with seeds of

species that are important in medicine [59, 118, 135, 136] and agri-

culture [11, 12, 13, 44, 47, 69, 96, 107]. Few such studies have been

done with seeds of forest trees, particularly the coniferous seeds

which have been used as the main seed sources for forest regeneration

throughout the world. Reports on sterols in these coniferous species

are very rare, possibly limited to less than a dozen [22, 111]. Thus,

the study of sterols in coniferous seeds could contribute to an under-

standing of tree physiology.

Longleaf pine, Pinus palustris, was selected for this study

because of the wide distribution of this commercially important species

in the southeastern portion of the United States. Also the seeds of

this species of known origin can be obtained in sufficient quantities,

permitting the design of properly controlled experiments.

The objectives of the present investigation were to study the pre-

sence and variation in amounts of sterols in longleaf pine seeds during

germination, the effects of inhibitors on sterol biosynthesis during germination, and to investigate the possible function(s) of sterols on growth and development of germinating seeds.

LITERATURE REVIEW

The study of sterols began with investigations on the dissolution
of gallstones [38]. Isolation of the main constituent of gallstones
from alcoholic solution was first carried out by Poulletier de la
Salle, circa 1769 [195]. Similar experiments were repeated by
Dietrich in 1788 [66] and DeFourcroy in 1789 [65]. In 1816, Chevreul
gave the name "cholesterine" (chole, bile; stereos, solid) to the waxy,
scaly substance obtained by digesting gallstones with alcohol [52].
With the discovery of "cholesterine", its occurrence in man and other
higher animals was investigated extensively. Berthelot in 1859 [36]
prepared esters of "cholesterine", establishing its alcoholic nature,
and in usage today this is indicated by the termination, -ol, thus,
cholesterol [38].

In parallel to numerous studies of cholesterol in animals, research
on plant sterols probably began with Braconnot (1811) [42] who iso-
lated from mushrooms an "adipocire" that Vauquelin (1813) [230] and
Gobley (1856) [102] termed "agaricine" and Tanret (1889) [218] named
"ergosterine" (ergosterol). In 1878, Hesse [126] prepared from
Calabar beans and peas a sterol indistinguishable from the "cholesterol"
that Beneke (1862) [26] had previously obtained from peas. Hesse [126]
observed that while it seemed to be isomeric with animal cholesterol,
it was clearly not identical with it. Because cholesterol had been
associated generally with animal sources, Hesse named the new vegetable
substance phytosterol. Later, Windaus and Hauth (1906) [234] proved
that the Calabar bean phytosterol prepared by Hesse was actually a mix-
ture of sitosterol and stigmasterol.

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Studies on sterol synthesis during germination of seeds started with the work of Schulze and Barbieri in 1882 [211]. They found that the sterol content of whole seedlings of lupine after germination in the dark was greater than that of the ungerminated seeds. In 1918, Ellis [86] reported that the main phytosterol present in whole wheat and in the embryo was sitosterol. Also the percentage of phytosterol present in the embryo was much higher than in the plant, suggesting an essential function of sterol in germination and growth. Since its discovery by Burian in wheat and rye embryo in 1897 [43], sitosterol had usually been regarded as a single entity. In 1926, Anderson et al. [2, 3] demonstrated that sitosterol exists in at least three isomeric forms, α, β, and γ, which are so intimately associated and so difficult to separate it is not surprising that they were considered as a single substance in the initial analysis.

In 1927, Terroine et al. [220] demonstrated that the sterol content of seeds usually increased during germination, even in the dark. They assumed that the sterols originated at the expense of fats stored in the seeds. Their assumption was verified in 1936 by MacLachlan [174] who observed that the germination of soybeans was accompanied by a marked reduction in total fat, followed by an increase in sterols. Also during this rapid mobilization and utilization of fat, esterification of the sterols showed a marked increase.

Up to the 1940's, no function had been ascribed to the universally distributed phytosterols in plants. In a series of papers published between 1943 and 1946, Balansard et al. [14, 15, 16, 17, 18, 19] showed that saponins, a group of steroids of wide occurrence in the plant kingdom, were able to elicit plant growth responses when added to

plants in certain stages of growth. The growth rate of isolated wheat embryos was approximately doubled by optimal concentrations of saponin, although higher concentrations were toxic [16]. Saponin applications also increased the rate of development of shoots and roots in begonia [14]. Treatment of seeds of cereals with dilute solutions of saponins accelerated germination and increased the subsequent rate of seedling growth [18]. Seeds of peas and corn also had increased water uptake in the presence of saponins, with a subsequent increase in the rate of germination [15, 19].

In the early 1950's, the important discovery that the steroid, cortisone, was of significant value in the treatment of arthritis and other diseases encouraged many chemists to undertake the arduous task of increasing this commercial product through partial synthesis from plant steroids such as ergosterol, stigmasterol, and diosgenin [35, 212]. This search for suitable steroids caused an increase in interest in the occurrence and distribution of steroids in plants. In 1953, Bergmann [35] compiled much of the information known on plant sterols. He listed the different sterols occurring in fungi, lichens, algae, and higher plants; details on their molecular formula, melting point, and specific rotation were also clearly described.

Although isotopic tracers were used by Arigoni in 1958 to follow the biosynthesis of sterols in germinating soybeans [4], his work was hampered by the lack of techniques to separate plant sterol mixtures. The sterols of such mixtures differ only in their small degree of unsaturation and in the shape of their side chain at C-24, showing such similarities in their solubilities as to make their separation difficult by conventional chromatographic techniques [35, 90, 112]. Since the report in 1960 by Beerthuis and Recourt [23] that gas-liquid chromato-

graphy (GLC) could be used for separation and partial identification of sterols, many studies using GLC techniques have been conducted on sterols in germinating seeds of different species [145, 200].

The application of GLC to isolation and identification of sterols has revealed the heterogeneous nature of many plant sterol fractions, some of which are too complex to resolve even by GLC [58, 171, 222, 227, 228]. Difficulties and problems are still evident when a positive identification of a particular plant sterol is desired [58, 204]. Some attempts have been made to use other techniques, such as mass spectrometry, to identity phytosterols, but the results obtained were not always satisfactory due to the difficulty in obtaining pure samples in sufficient quantities for an analysis [153, 167]. Knights in 1967 [153] used a combination of GLC and mass spectrometry for identification of fifteen plant sterols and closely related compounds. Since then, more advances and improvements have been made in the extraction and identification of sterols in different plant species [44, 47, 69], facilitating the studies of their possible functions in the plants in which they occur.

Biochemical Aspects

It has now become clear that the de novo biosynthesis of sterols occurs essentially by the same pathways regardless of the organism in which it takes place [183]. It is generally accepted that mevalonic acid (MVA) serves as the first intermediate of isoprenoid biosynthesis in mammals [219], higher plants [13, 184, 226], algae [100, 101], and fungi [216]. Langdon and Bloch in 1953 [164, 165] demonstrated that squalene was a sterol precursor in animals. Later, the conversion of MVA-2-^{14}C to squalene was demonstrated in germinating seeds of Pisum sativum [48, 49], and that squalene is the possible sterol precursor in higher plants [27].

In theory, all trans-squalene may be the starting material from which cyclic triterpenes will be formed [115]. Both plants and animals convert squalene to squalene 2,3-oxide [31, 199, 233] from which there are two major routes by which steroids are biosynthesized, through lanosterol and cycloartenol [115, 118]. The former appears to operate in animals [40] and fungi [231], while in plants the first cyclic product is often cycloartenol [54, 87, 180, 197]. The conclusion that sterols are synthesized via lanosterol in animals and via cycloartenol in plants comes from the fact that lanosterol has been isolated from plants less often than has cycloartenol [114]. The incorporation of labeled acetate and MVA into cycloartenol rather than lanosterol, both in vivo and in vitro, has been demonstrated in many plant species using both photosynthetic and nonphotosynthetic tissues [5, 150, 151, 175, 196, 202]. Cycloartenol also appears to be the key triterpene in algae since they incorporate MVA and squalene 2,3-oxide into this triterpene rather than into lanosterol [95, 100, 190, 198, 201].

At one time, lanosterol was thought to be a precursor in plant sterol biosynthesis; this came from the reports of isolation of lano- sterol from tobacco [32] and Euphorbia latex [193]. Recent studies have shown that the component that was thought to be lanosterol in tobacco is 24-methylene cycloartenol [33]. The Euphorbia latex con- tains both cycloartenol and lanosterol, but lanosterol is not actually found in the plant tissue and is considered an end-product in latex [194]. Labeled cycloartenol is converted to lanosterol, but not vice versa, in Euphorbia latex, and the biosynthesis of triterpenes in the latex is independent of that in plant tissue [194].

The last phases in sterol biosynthesis are the conversion of cycloartenol to different sterols. Although cholesterol has been found

to be distributed in small amounts in higher plants [104], the major plant sterols that contain additional alkyl groups at C-24 have been identified as sitosterol, campesterol, and stigmasterol [104, 111]. There are many reports of incorporation of labeled acetate or MVA into different sterols in different plant species [4, 13, 29, 30, 135, 138, 139]. When seeds of Pinus pinea were germinated in presence of MVA-2-^{14}C, a high yield of labeling in campesterol, isofucosterol, and sitosterol was obtained [226].

The conversion of cycloartenol to the major sterols requires introduction of an alkyl group at C-24, demethylations at the C-4 and C-14 positions, opening of the 9β-19-cyclopropane ring, and introduction of the Δ^5 bond [111]. The introduction of an alkyl group at C-24 does not occur for cholesterol.

In the alkylation process, the C-28 and C-29 of the major plant sterols are derived from S-adenosyl methionine, either by transmethylation for C-28 (campesterol) or double transmethylation for both C-28 and C-29 (sitosterol and stigmasterol) [10, 51, 167, 236]. Although the exact stage and mechanism of alkylation is still not completely understood [114], the intermediate which is alkylated must have a double bond at C-24 position [205]. This is substantiated by the presence of 24-methylene cycloartanol [1, 34, 122, 181, 192], 24-methylene lophenol, and 24-ethyldiene lophenol in numerous plants (99). The 24-methylene cholesterol, differing from 24-methylene lophenol by a double bond at C-7 and an extra methyl group at C-4, has also been known as a key intermediate in the biosynthesis of C-28 and C-29 sterols [20, 118, 225]. Reduction of the 24(28) double bond produces either 24 α-methylcholesterol (campesterol) or 24β-methylcholesterol (22-dihydrobrassicasterol). When a second methyl group is introduced into methylene cholesterol,

fucosterol or isofucosterol is formed. Reduction of the 24(28) bond of this fucosterol will produce 24α-ethylcholesterol (sitosterol) [118]. These processes have been shown to occur in pine tissues. When the germinating seeds of <u>Pinus pinea</u> were incubated with $28\text{-}^{14}C$-24-methylene cholesterol, there was labeling in campesterol, isofucosterol, and sitosterol [226].

The incorporation of $MVA\text{-}2\text{-}^{14}C$ into triterpenes and sterols of various plants indicates that, as in lanosterol demethylation in animals, the loss of the 4α-methyl group is followed by epimerization of the 4β-methyl to the 4α - position before removal [97, 150, 151]. The isolation of many plant triterpenes lacking a methyl group at C-4 indicates that the overall demethylation sequence in plants may be C-4α ⟶ C-14α ⟶ C-4β (after epimerization), rather than that visualized as operative in rat liver homogenates, C-14α ⟶ C-4α ⟶ C-4β (after epimerization) [98, 180].

In the final stages of sterol biosynthesis, a rearrangement of the double bonds must occur. Although the sequence of these arrangements has not been well established, the possible pathway would be Δ^{7}⟶ $\Delta^{5,7}$⟶ Δ^{5}, and these steps seem to be irreversible [99, 111].

The possible general pathway for biosynthesis of free plant sterols is shown in Fig. 1.

In higher plants, sterols have been found to occur in three different forms: free sterols, steryl esters of fatty acids, and steryl glycosides which are acylated and non-acylated [111, 118]. The esters of major plant sterols have been isolated from different tissues of many plant species [44, 143, 146, 149, 235], as well as from cellular organelles [144]. The most important acid components of the steryl esters have been shown to be palmitic, oleic, linoleic, and
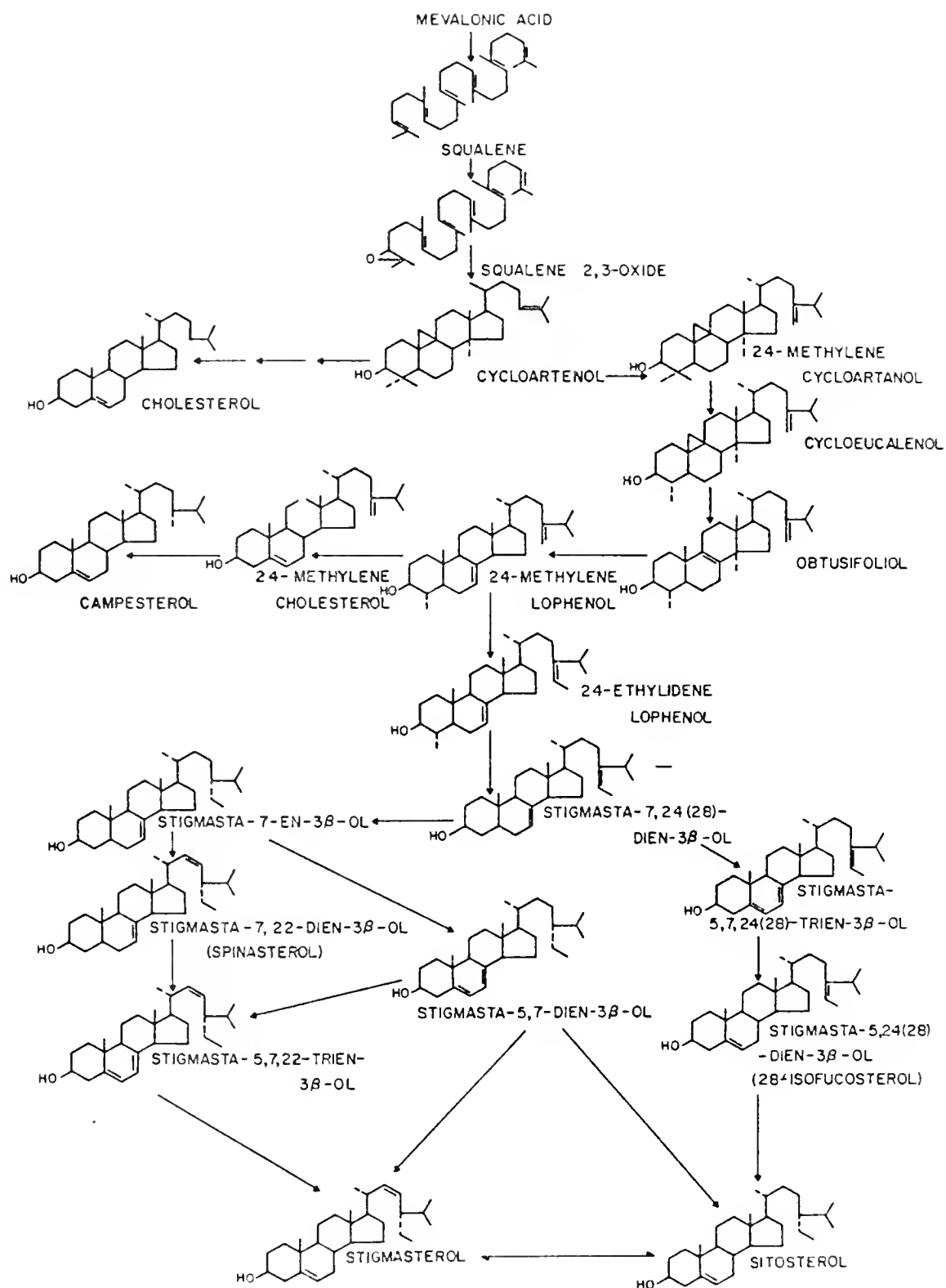
Figure 1. A possible biosynthetic pathway of plant sterols.
Adapted from [101, 111, 175, 181].

linolenic acid [44, 186]. Although steryl esters have been isolated from plants, their biosynthetic pathway is still in question, and no localization for esterification has been established [111]. When ^{14}C-labeled MVA was used as a substrate, the radioactivity recovered in the steryl esters was much higher than when six-day-old tobacco seedlings were incubated with ^{14}C-labeled cholesterol or sitosterol [45, 46].

The isolation of steryl glycosides in both forms has also been reported from a variety of plants [44, 46, 88, 169]. Any 4-demethyl sterol such as sitosterol, campesterol, stigmasterol, and cholesterol can be the moiety of both steryl glycosides [46, 169]. The sugar components have also been shown to be commonly glucose and in some cases mannose and galactose [81, 82, 147, 169, 221, 237], and the main acyl moieties in the acylated forms are palmitic, stearic, oleic, linoleic, and linolenic acid [147, 169, 221]. The biosynthesis of steryl glycosides has been obtained by particulate enzyme preparations from seeds of different species [46, 131, 132, 148, 191]. The most active glycosyl donor is UDP-Glucose, and the reaction is stimulated by ATP [78, 111, 132]. The pH of the incubation medium varies, depending on tissues [79, 80], but optimal conditions are near pH 7.0 [46, 187].

Two different pathways have been proposed for the formation of acylated steryl glycosides: through steryl glycoside or acyl glycoside [111]. Experiments with a cell-free system support the former [9, 132] while in vivo experiments favor the latter pathway [46, 82].

Physiological Aspects

The almost universal occurrence of sterols in plants would suggest that these compounds have some definite role in plant metabolism. As yet the nature of this function is quite obscure, and little physio-

logical significance has been attached to the universally distributed phytosterols [44, 118].

In animal systems, sterols serve at least three functions: as membrane components, as hormones, and as precursors of other steroids [111, 117, 120]. It has been suggested that sterols may act in a similar manner in both plants and in animals [117, 120].

Sterols as Membrane Components

The role of sterols as components of plant cell membranes was suggested in 1918 by Ellis [86]. Recently this role of sterols was re-emphasized by many authors [44, 105, 107, 109, 130, 144, 145]. In general, the level of sterols was reported to increase with time of germination in many plant species, leading to the suggestion that actively growing tissues, such as germinating seeds and developing seed-lings, accumulate sterols as a result of increased membrane synthesis [44, 111, 145].

In a study of the distribution of sterols in organelles, it was found that all membranes contain fractions of free sterols, steryl glycosides, and steryl esters [107, 144]. Furthermore, when cholesterol - ^{14}C was added to the isolation medium, it became distributed intracellu-larly, leading to the conclusion that significant amounts of sterols in plants are associated with membranes, including those of organelles, and that sterols play an important role as structural and functional components [107, 111].

The forms and categories of sterols that may constitute an inte-gral part of the lipid layer of the cellular membranes are topics of many discussions [111, 130, 145]. Grunwald [105] noticed that choles-terol was more effective than $CaCl_2$ in preventing the leakage of β-

cyanin from red beet root cells treated with methanol. Other sterols, such as β-sitosterol and stigmasterol, were less effective. Cholesterol was also found more effective than the other sterols in restoring the K^+ and NO_3^- uptake capability in etiolated Pisum sativum stem sections treated with filipin [124]. For membrane stabilizing effectiveness, cholesteryl palmitate and cholesteryl glycoside, both lacking a free hydroxyl group at the C-3 position of the cholestene nucleus, were found ineffective in preventing electrolyte leakage from barley root cells, whereas free cholesterol was effective in this regard [109]. Also, when plant sterols were permitted to incorporate in vitro into erythrocyte membranes, it was found that cholesterol entered more readily than sitosterol, while campesterol was intermediate. Campesterol and sitosterol contain an extra methyl and ethyl group in the chain, respectively, which could reduce the ability of a sterol to enter the phospholipid membrane. Also, the presence of the double bond in the side chain of ergosterol and stigmasterol apparently reduces their flexibility, resulting in a reduction of their capacity to dissolve into the erythrocyte phospholipid complex [22, 77].

All these facts have led to the suggestion that only sterols having a free hydroxyl group at the C-3 position and a flat molecular configuration similar to cholesterol could be absorbed into the membrane phospholipids and be active [110, 111]. The level of free sterols which has been reported to increase with time of germination in seed of a number of species may partially support this suggestion and may explain the possible function of free sterols in higher plants [44, 69, 145].

Information on the physiological role of steryl esters and steryl glycosides is very limited. By analogy with cholesteryl esters in

animal tissues, sterols were postulated to be transported intracellu-
larly as esters from their site of synthesis to the various organelles
[145]. The steryl glycosides probably represent either storage or
transport forms of sterols in plants [8, 132, 224]. The addition of a
sugar moiety to the nonpolar free sterol facilitates its solubility in
the cytoplasm for possible transport to different parts of the develop-
ing seedlings [132].

Sterols as Plant Hormones

Sterols may have hormonal activity in plants as well as in animals
[117]. Through oxidation and aromatization, plant sterols were reported
to be transformed to other steroids which have been identified as
hormones in animals [22, 28, 111, 116, 120, 207].

Studies on steroid hormones in higher plants so far have been
largely concerned with their function in the insect molting process
[22, 116]. Recent isolations of sterols with insect molting hormonal
activity contributed to the proof of the capacity of steroid biosynthesis
in higher plants which in many ways resembles that in animals [121,
127].

The status of our knowledge of the influence of animal steroid hor-
mones on growth and reproduction of plants began in 1945 with Löve and
Löve [172]. They found that they could produce male or female flowers
on Melandrium dioecum at will by applying either androgens or estrogens
to the stems before flowering.

In 1971, Gawienowski et al. [94] reported that treating monoecious
cucumber plants with either 17β-estradiol or testosterone caused the
induction of pistillate flowers. An increase in estrogen biosynthesis
during flowering in Phaseolus vulgaris was also reported in the same
year by Kopcewicz [160]. Quantitative investigations of the content of

the estrogen substance in the developing bean plants showed that this compound appears as flower buds emerge, reaching a maximum at the period of flower bud development and pod formation. Estrogens increased the number of flowers when applied to Ecballium elaterium [161] and Cichorium intybus [158]. These sex hormones also influenced the female to male sex ratio in favor of female flowers, while androgens changed the sex ratio in favor of maleness [161]. In addition to the effect of animal steroids on flowering in plants, the major plant sterol, sitosterol, was also active in the initiation of flower buds in Chrysanthemum [39].

The suppression of floral induction by steroid biosynthesis inhibitors was reported in Pharbitis nil [41, 206], Xanthium pensylvanicum [41], and Lolium temulentum [89]. The animal steroid inhibitor SKF 7997-A_3 inhibits effectively the flower-inducing processes only if applied to the leaves and shortly before the beginning of the dark period in two short-day plants Xanthium and Pharbitis. Application to buds or to leaves after a long night is without effect. Since the substance is a steroid biogenesis inhibitor in animal tissues and since it also blocks sitosterol and stigmasterol biosynthesis in Xanthium leaves, there may be participation of steroidal substances in floral induction and sex expression in plants. However, their mechanism of action has not been determined. It has been suggested that steroids may constitute one of the components of the hypothetical flowering hormone [41, 160].

Besides the effect of steroids on flowering and sex expression, there are also additional responses on plant growth [123, 178]. Sitosterol, estrone, and 17β-estradiol were reported to stimulate growth in six-day-old dwarf seedlings of Pisum sativum [155]. Also sitosterol,

stigmasterol, and cholesterol, like gibberellic acid, could overcome completely the effect of the retardant Amo 1618 on stem growth of tobacco seedlings [67]. It had been shown that estrone increased the endogeneous level of gibberellins in dwarf Pisum sativum [156]. Furthermore, auxin content of the seedlings of Pinus silvestris and Pisum sativum also showed a sharp increase over the control when both were treated with estrone and 17β-estradiol [157, 159]. This indicates the possibility that steroids by themselves do not act as hormones, but may have an effect on the biosynthesis of gibberellins which, in turn, influences flowering and plant growth [158, 159].

Sterols in Developing and Germinating Seeds

The presence and accumulation of sterols during seed development were reported in such important agronomic crops such as soybean [141], pea [12], and corn [63, 64]. In general, the biosynthesis of sterols was highest in the least mature seeds and decreased with seed maturity, with formation of β-amyrin as a final regulation of sterol biosynthesis in mature seeds [12].

During corn seed development, free sterols and steryl esters were the major sterol fractions in both low and high oil content varieties. Steryl glycosides accounted for only a small portion of the total sterol content [63, 64]. Also in corn, while the free sterol level rapidly decreased between 15 and 26 days after pollination, the steryl esters continued to increase, reached a maximum level at the late stages of linear kernel growth, and then decreased during the final part of the growth period [63].

In soybean, during seed maturation the free form of sterols was the most abundant and amounted to 70% of the total sterols [141].

Steryl esters and steryl glycosides also accumulated during maturation, but free sterols accumulated for a period longer than that for steryl glycosides and steryl esters.

The presence of sterols in mature dry seeds was also reported [12, 73, 133]. In the case of peas, β-sitosterol was present at a concentration as high as 0.67 mg/g of dried seed [12]. It is possible that large amounts of sterols are synthesized during seed development and accumulate in dry mature seeds to satisfy the initial demands made by growth during germination, probably either by production of new membranes or transformation of the existing membranes to be more permeable to gases and water [12, 111].

Sterol synthesis was not found to occur to any significant extent during the first 2 to 4 days following germination [12, 44, 69, 145]. Perhaps sterol reserves were sufficient in early stages of germination and biosynthesis was required only after reserves were depleted [12, 111].

As previously discussed, an increase in total sterol content was observed in different species during germination [44, 69, 70, 71, 72, 135]. This increase in sterols was probably a result of increased cellular organelle production and new membrane formation [44, 111, 145]. Sterol biosynthesis was also proportional to an increase in dry matter and protein, and was higher in seedlings grown in nutrient solution than those grown in distilled water [68, 69].

Sterols as Protective Agents

It has been suggested that sterols could be used to control fungi and insects [22]. When the cotton leafworm insect was grown on a medium containing β-sitosterol, a high level of insect sterility was

observed, suggesting that the use of naturally occurring plant sterols may be valuable as a means of biological control [208]. Reports also showed that sterols may cause an inhibition in growth and reproduction of some fungi [84]. Thus, the presence and level of sterols in plant tissues could play a role in disease resistance and may be involved in affording plant protection against fungi and insects [6, 117, 125, 137]. By manipulation of the sterol content of the host through breeding, more resistant crops may be obtained [85]. The application of methodology for altering the sterol level in plants could lead to more efficient crop production and could assist in the response to the demands of the world population for increased food productivity [22].

MATERIALS AND METHODS

Longleaf pine seeds were germinated under controlled conditions in a growth chamber, and the germinating embryos and young seedlings were used for extraction of sterols, reducing sugars, and chlorophyll. The effects of inhibitors on the metabolism of germinating pine seeds were also studied. All chemical determinations were made in duplicate, and the results reported were means having reproducibility within ± 5%.

Chemicals

Cholesterol, stigmasterol, 5 α-cholestane, digitonin, trisodium DL-isocitrate, sodium glyoxylate, Tris-HCl, Tris-Base, and cycloheximide were purchased from Sigma Chemical Company. Campesterol, β-sitosterol, the U-tube glass column, and the packing material OV-101 5% coated on Gas-Chrom Q 80-100 mesh were purchased from Applied Science Lab., Inc. Solvents were reagent grade and were obtained from Fisher Scientific Co.

Tris-(2-diethylaminoethyl)-phosphate trihydrochloride (SKF 7997-A$_3$) was obtained from Smith, Kline, and French Laboratories. Abscisic acid was obtained from the Department of Fruit Crops, University of Florida. Mercuric acetate was purchased from Fisher Scientific Co. Silica gel for column chromatography was from Baker Chemical Co. Radioactive ^{14}C-cholesterol, ^{14}C-cholesteryl palmitate, and ^{3}H-cholesterol were purchased from New England Nuclear Company.

Sources of Seeds

Longleaf pine seeds (_Pinus palustris_ Mill.) were purchased from

Resource Operations, Inc., Birmingham, Alabama. The seeds were collected in 1973 in the region of Escambia County, Alabama. The percentage of germination was approximately 70%. Seeds were stored in a cold room at $5^{o}C$ until used.

Preparation and Germination of Seeds

Seeds were soaked in double distilled water under aerated conditions in a cold room ($5^{o}C$) for 24 hours. They were planted 1.5 cm deep in moist vermiculite in glass trays and then placed in a growth chamber at $25 \pm 1^{o}C$ and a 12-hour day (fluorescent light 400 ft-c). The seeds were checked daily and double distilled water added, if necessary, to ensure adequate moisture. Many seeds started to germinate (radicle protrusion) on the 5th day of incubation.

Seeds showing radicle protrusion were selected and used for experiments. These seeds were considered to be in the 1st day of germination and were placed in moist vermiculite and returned to the growth chamber. At the end of each predetermined period of growth and development, the seeds or seedlings were removed from vermiculite, washed with double distilled water, and only those having approximately the same length of radicle were used. The germinating embryos were removed from the megagametophytes, washed with double distilled water, and used for sterol extraction.

Sterol Extraction and Fractionation

An extraction technique based on the procedures of Stedman and Rusaniswkyi [215] and Keller, Bush and Grunwald [44, 107, 142] was used. The germinating pine embryos were homogenized in 5 ml acetone with an Omni-mixer for 5 minutes at full speed. A small amount of fine glass beads was added to facilitate homogenization. The result-

ing homogenate was extracted with 200 ml acetone in a Soxhlet apparatus for 24 hours. The acetone extract was cooled to room temperature and divided into two equal aliquots. The first aliquot was used for extraction of total sterols, and the second aliquot was used for fractionation of three different forms of sterols.

For total sterols, the acetone extract was taken to dryness under vacuum, and 50 ml of 95% ethanol containing 0.15 ml concentrated H_2SO_4 were added and refluxed for 15 hours to cleave the steryl glycosides. Fifteen ml of 10% KOH in 95% ethanol (w/v) were then added and the solution was refluxed for 30 minutes to hydrolyze the steryl esters.

The resulting mixture was cooled to room temperature and was neutralized to pH 7.0 with H_2SO_4 - ethanol solution. The sterols were then extracted three times with 30 ml of n-hexane. A small amount of double distilled water was added in the first extraction to give two separate layers in the separatory funnel. The n-hexane fractions were combined and extracted three times with 50 ml of 90% methanol. The methanol fraction was then back extracted twice with 30 ml of n-hexane. All the n-hexane fractions were combined and taken to dryness under vacuum. The resultant residue was dissolved in 20 ml boiling absolute ethanol and transferred to a centrifuge tube in a 100°C water bath. Ten ml of hot 2% digitonin in 80% ethanol (w/v) were added to each tube, and the bath was boiled continuously for a few minutes, after which 5 ml of hot double distilled water were added, and the mixture allowed to cool and remain overnight at room temperature.

The tubes were centrifuged at 15,000 g for 30 minutes and the precipitates were washed three times with 30 ml of 80% ethanol and three times with 30 ml of diethyl ether. The white digitonide precipitates were dried overnight at room temperature, and then were hydrolyzed at

$70^{\circ}C$ for 2 hours with 2 ml of pyridine, containing a known amount of 5 α-cholestane as an internal standard. The pyridine mixture was left at room temperature for 12 hours, and the digitonin was removed by precipitation with 30 ml of diethyl ether followed by centrifugation at 10,000 g for 30 minutes. The ether layer was recovered, taken to dryness under an air stream, and the residue dissolved in ethyl acetate for injection into a gas-liquid chromatograph.

The second acetone aliquot was also taken to dryness under vacuum; 50 ml of 95% ethanol were added and partitioned three times against an equal volume of n-hexane. The ethanol fraction contained the steryl glycosides, and the free sterols and the steryl esters were in the n-hexane fractions. The method of Goodman [103] was used for separation of free sterols and steryl esters. A 2 x 10 cm glass column was packed with 5 g of silica gel (70-325 mesh) and the n-hexane fraction was applied as a slurry to the column. A serial elution was carried out as follows: 50 ml of n-hexane followed by 50 ml of 10% benzene in n-hexane (discarded); 100 ml of 40% benzene in n-hexane (steryl esters); 50 ml of benzene (discarded); and 100 ml of chloroform (free sterols).

The glycosidic, esterified, and free sterol fractions were dried under vacuum. The glycosidic fraction was refluxed for 15 hours in 50 ml of 95% ethanol containing 0.15 ml of concentrated H_2SO_4, neutralized with KOH-ethanol, and partitioned three times against n-hexane. The esterified fraction was hydrolyzed for 30 minutes in 15 ml of 10% KOH in 95% ethanol (w/v), neutralized with H_2SO_4-ethanol, and partitioned three times against n-hexane. The hexane fractions were dried under vacuum. These fractions were transferred to plastic centrifuge tubes with boiling absolute ethanol and precipitated with digitonin as described above to obtain free sterols.

Radioactive ^{14}C-cholesterol was used for determination of the
recovery of the total sterol extraction. ^{3}H-cholesterol and [14]C-
cholesteryl palmitate were added to the silica gel column for correc-
tion of any losses through fractionation. Radioactivity was measured
on a Packard Tri-Carb liquid scintillation spectrometer.

The overall flow chart of procedures used for extraction and
fractionation of longleaf pine sterols was summarized in Fig. 2.

Identification of Sterols

The sterol analysis was performed with a Packard Gas Chromatograph
Series 7300. The GLC system consisted of a dual flow controller,
Model 824, equipped with a flame ionization detector; a "U" column air
oven console, Model 805; a deviation temperature controller, Model 873;
a dual electrometer, Model 843; a dual bipolar high voltage supply,
Model 834; and a recorder, Model 562. The column used was a 6-foot
glass U-tube with a 3.5 mm i.d. packed with Gas-Chrom Q, 80-100 mesh,
coated with 5% dimethyl silicone liquid OV-101 [106, 108]. Helium was
the carrier gas at a flow rate of 100 ml/minute at 30 psi of column
inlet pressure. The flow rates of hydrogen and air were 100 ml/minute
and 350-400 ml/minute, respectively.

The column was operated isothermally at $250^{o}C$ and the temperatures
of the injector and detector were $275^{o}C$. The internal standard 5 α-
cholestane was chromatographed with each sample and the retention times
of sterols were determined relative to cholestane. For quantitative
analysis of individual sterols, peak areas were measured. Total sterol
values were summations of the individual sterol values.

The GLC-mass spectrometry of the different sterol components was
performed with a combination of a double-beam, double-focusing AEI

GERMINATING PINE EMBRYOS

| HOMOGENIZE IN ACETONE WITH OMNI-MIXER

(5 MINUTES)

HOMOGENATE

EXTRACT IN SOXHLET APP. WITH ACETONE

(24 HOURS)

ALIQUOT 1 — ALIQUOT 2

ALIQUOT 1:
* DRY UNDER REDUCED PRESSURE
* ADD 50 ML 95% ETOH + 0.15 ML H_2SO_4
* REFLUX FOR 15 HOURS

ALIQUOT 2:
* DRY UNDER REDUCED PRESSURE
* ADD 50 ML 95% ETOH
* PARTITION AGAINST n-HEXANE

EXTRACT SOLUTION

ETOH FRACTION
(STERYL GLYCOSIDES)

* ADD 15 ML 10% KOH IN 95% ETOH
* REFLUX FOR 30 MINUTES
* NEUTRALIZE WITH H_2SO_4-ETOH

TOTAL FREE STEROL HYDROLYSATE

HEXANE FRACTION

* PARTITION AGAINST n-HEXANE

COLUMN CHROMATOGRAPH

HEXANE FRACTION

(5g SILICA GEL)

* DRY UNDER REDUCED PRESSURE
* PRECIPITATE WITH DIGITONIN
* COOL OVERNIGHT AT ROOM TEMPERATURE

FREE STEROLS   STERYL ESTERS

STEROL DIGITONIDE

* WASH WITH 80% ETOH AND ETHER
* DRY OVERNIGHT AT ROOM TEMPERATURE

DRY STEROL DIGITONIDE

* ADD 2 ML PYRIDINE, HEAT AT
  70°C FOR 2 HOURS
* LEAVE OVERNIGHT AT ROOM TEMPERATURE
* REMOVE DIGITONIN WITH ETHER

STEROLS IN ETHER

* DRY UNDER AIR STREAM
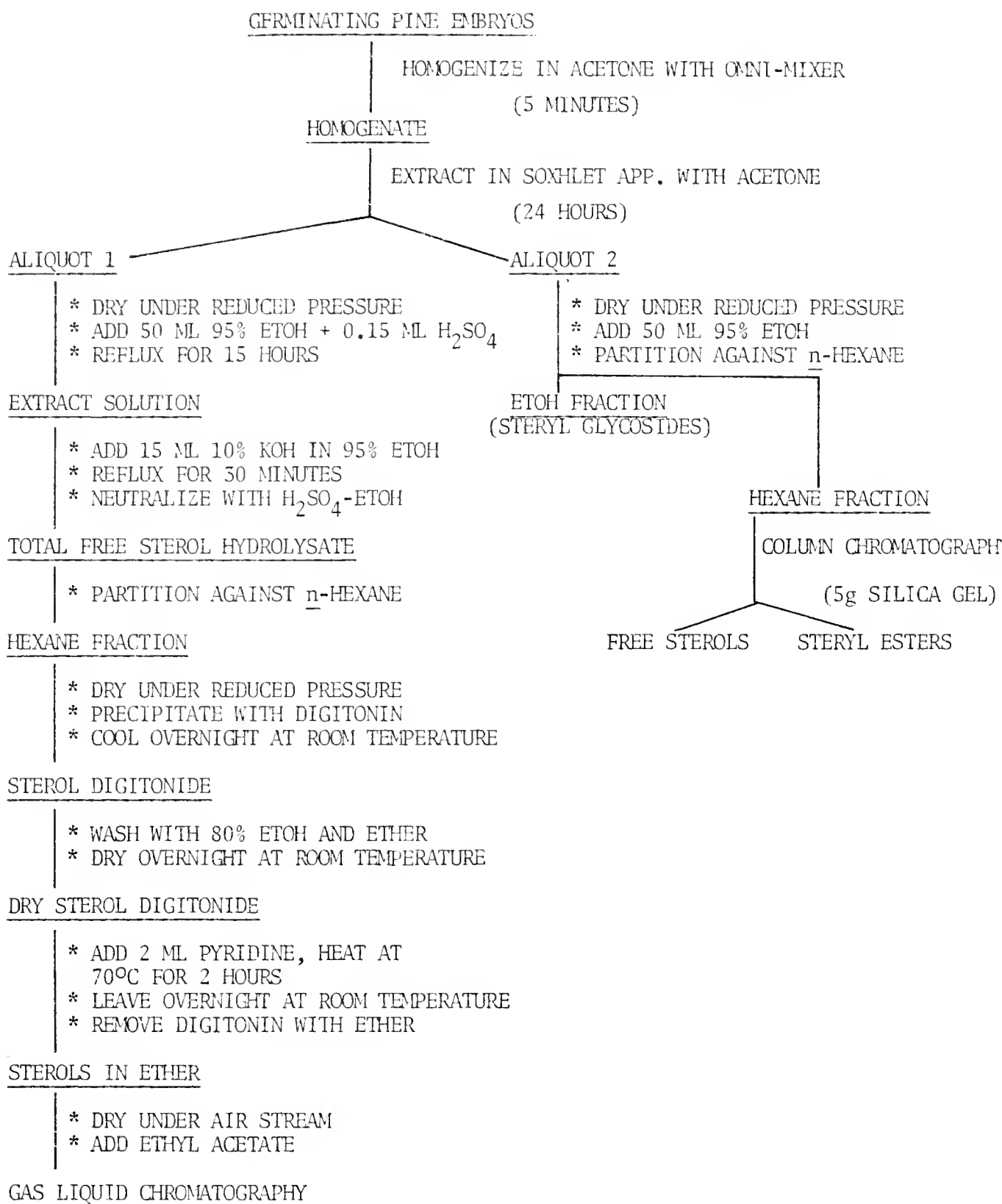* ADD ETHYL ACETATE

GAS LIQUID CHROMATOGRAPHY

Figure 2. Flow chart for procedures used in the extraction and fractionation of longleaf pine sterols.

MS-30 Mass Spectrometer coupled by a silicone membrane separator to a Pye Gas Chromatograph. The GC was fitted with a 5-foot glass column with 6.4 mm o.d. packed with Supelcoport 100-120 mesh coated with 3% methyl silicone SP 2100. The column was operated isothermally at 270°C, and helium was used as carrier gas at 30 ml/minute. The ion source was operated at 220°C, and the electron beam was at a potential of 70 eV. An AEI DS-30 digital computer which was hooked up to the combined GLC-MS system was employed to acquire and process the data.

Effects of Inhibitors on the Biosynthesis of Sterols, Reducing Sugars, and Chlorophyll

The following inhibitors were tested on germinating pine seeds for their effects on biosynthesis of sterols, reducing sugars, and chlorophyll: tris-(2-diethylaminoethyl)-phosphate trihydrochloride (SKF 7997-A_3), abscisic acid (ABA), and cycloheximide. The inhibitors were dissolved in double distilled water. The pH's of the solutions were immediately adjusted to 7.2-7.4 either with 0.1 N HCl or 0.1 N NH_4OH [41, 178]. The final concentrations of the inhibitors to be tested were SKF 7997-A_3 5 mg/ml; ABA 0.25 mg/ml; cycloheximide 0.25 mg/ml.

The seeds were prepared and germinated as mentioned above. Only the seeds showing radicle protrusion were selected. The seeds were immersed in the solutions (200 seeds/100 ml) under aerated conditions for 30 minutes. The seeds were then removed, blotted, planted in moist vermiculite and placed in growth chamber under the same conditions as stated above. At the end of 3, 7, and 12 days, the germinating seeds and seedlings were removed and washed with double distilled water. The embryos were separated from the megagametophytes, washed with double distilled water, and used for extraction and determination of total sterols, chlorophyll content, and reducing sugars.

Sterol Extraction and Identification

The total sterols were extracted and identified by GLC as described above. The different sterol components were also verified by GLC-MS to ensure that there was no change in the identity of the different sterols.

Determination of Reducing Sugars

The samples were dried in an oven at 70°C for 48 hours, then ground to pass a 60-80 mesh screen and stored at 70°C in open glass vials until the plant material came to a constant weight. The vials were transferred to a desiccator to cool. Approximately 2-g samples were extracted overnight in a Soxhlet apparatus with 80% ethanol. The ethanol extract was taken to a syrupy stage under vacuum, and approximately 50 ml of double distilled water were added. The water solution was filtered under vacuum in a Buchner funnel using Whatman No. 1 filter paper and a double layer of activated charcoal and cellulose powder. The clear aqueous solution was brought to 100 ml with double distilled water, and the reducing sugars were determined according to a modified method of Nelson [57, 129, 182]. The transmissions were read at 500 nm [129] in a Beckman DB spectrophotometer, and the amounts of reducing sugars in the samples were calculated on a dry weight basis.

Chlorophyll Determination

Total chlorophyll was extracted and content calculated by a modified method of Starnes and Hadley [214]. Approximately 5 g of germinating embryos were macerated for 10 minutes at full speed in an Omni-mixer in 20 ml of 80% aqueous acetone and vacuum filtered through a Buchner funnel using Whatman No.1 filter paper. The residue was homo-

genized a second time for 5 minutes and refiltered to ensure that all chlorophyll had been extracted. The filtrate was brought to 100 ml and allowed to incubate for 1 hour. A 10-ml aliquot was taken and centrifuged at 2,500 g for 5 minutes. The absorbance of the supernatant was measured at 663 nm and 645 nm with a Beckman DB spectrophotometer. The concentrations of chlorophyll a (C_a) and b (C_b) , respectively, were calculated by the formulas

$$C_a = 12.717 \times A_2 - 2.584 \times A_1 = \text{mgs chl. a/liter}$$

$$C_b = 22.869 \times A_1 - 4.670 \times A_2 = \text{mgs chl. b/liter}$$

where A_1 and A_2 were the absorbances at wavelengths 645 nm and 663 nm, respectively.

These values were then used to determine the total chlorophyll content on a dry weight basis.

Isocitrate Lyase Assay

Germination of Seeds

Seeds were soaked in a cold room (5^OC) in double distilled water under aerated conditions for 36 hours. The seed coats, inner membranes, and nucellar caps were removed, and the decoated seeds were planted in moist vermiculite and transferred to a growth chamber for germination. The seeds were maintained at 25 ± 1^OC under a 12-hour day (white, fluorescent tubes, 400 ft-c). Most of the seeds started to germinate (radicle protrusion) on the second day. The germinating seeds were removed from the vermiculite and washed with double distilled water. The germinating embryos were removed and the megagametophytes were used for enzyme studies.

For certain experiments, the megagametophytes were split lengthwise, and the embryos were removed and discarded at 0 day and at 2 days.

Extraction and Assay of the Enzyme

Isocitrate lyase was extracted according to the method of Ching [53]. A sample of 10 gametophytes was ground in 10 ml of 0.05 M Tris-HCl buffer, pH 7.5, containing 10 mM mercaptoethanol and 1 mM disodium EDTA. The extract was centrifuged at 30,000 g for 10 minutes and the supernatant treated with charcoal.

The enzyme was assayed in a total volume of 1 ml (0.1 ml of the extract and 0.9 ml of a mixture of 0.10 M Tris-HCl buffer pH 7.4, 10 mM cysteine hydrochloride, 10 mM $MgCl_2$, and 20 mM trisodium DL-isocitrate) at 30°C for 5 minutes. Glyoxylate was determined by the mercuric acetate method of Jacks and Alldridge [134] with a comparable reaction mixture without substrate for the blank. Protein was extracted by a modified method of West [232] and was estimated by the method of Lowry et al. [173].

Steroid Inhibitor Treatment

After removing the seed coats and nucellar caps, the seeds were dipped in the solution of SKF 7997-A_3 (5 mg/ml, pH 7.2-- 7.4) for 30 minutes under aeration. The seeds were then blotted and planted in moist vermiculite under the germination conditions described above until harvested for enzyme extraction.

Sterol Treatment

A mixture of sterols (65% β-sitosterol and 35% campesterol) at a concentration of 5 mg/ml was used. The sterols were dissolved in 0.1% Tween 80 before adding distilled water [159]. The decoated seeds, after treatment for 30 minutes with the steroid inhibitor SKF 7997-A_3, were blotted and submersed in the sterol solution under aeration for another 30 minutes. The seeds were then removed, blotted, placed in

moist vermiculite, and transferred to a growth chamber for germination.
The isocitrate lyase was extracted and assayed at the end of each de-
sired period.

RESULTS

Identification of Sterols Isolated from Germinating
Pine Embryos

A typical GLC retention pattern and characterization of sterols isolated from 11-day-old longleaf pine seedlings are shown in Fig. 3 and Table 1. The relative retention of the two major peaks present in the total sterol fraction corresponded to the standards campesterol (peak 4) and β-sitosterol (peak 6), and the two minor peaks to the standards cholesterol (peak 3) and stigmasterol (peak 5). Peak 2 did not correspond to any available authentic sterol.

The mass spectrum data are presented in Tables 2, 3, and 4. The GLC peaks 4, 5, and 6 which corresponded respectively to campesterol, stigmasterol, and β-sitosterol had the m/e values specific for these sterols. These m/e values also corresponded to the ions obtained with the authentic standard sterols (Tables 2, 3, and 4) and with those published by Knights in 1967 [153].

The GLC peak 2 did not have any significantly high m/e values from 227 to 428. Most of the m/e ions in this region came from background values having intensities smaller than 1%. The most intense peaks were at m/e 225, 120, 106, 91, 77, and 57, having relative intensities 26%, 100%, 21%, 13%, 22%, and 13%, respectively. Based on these observations, it was concluded that the GLC peak 2 was not a sterol but probably was an unknown long chain hydrocarbon.

The GLC peak 3, which corresponded to the relative retention of the standard cholesterol, also did not show any high relative intensity

31
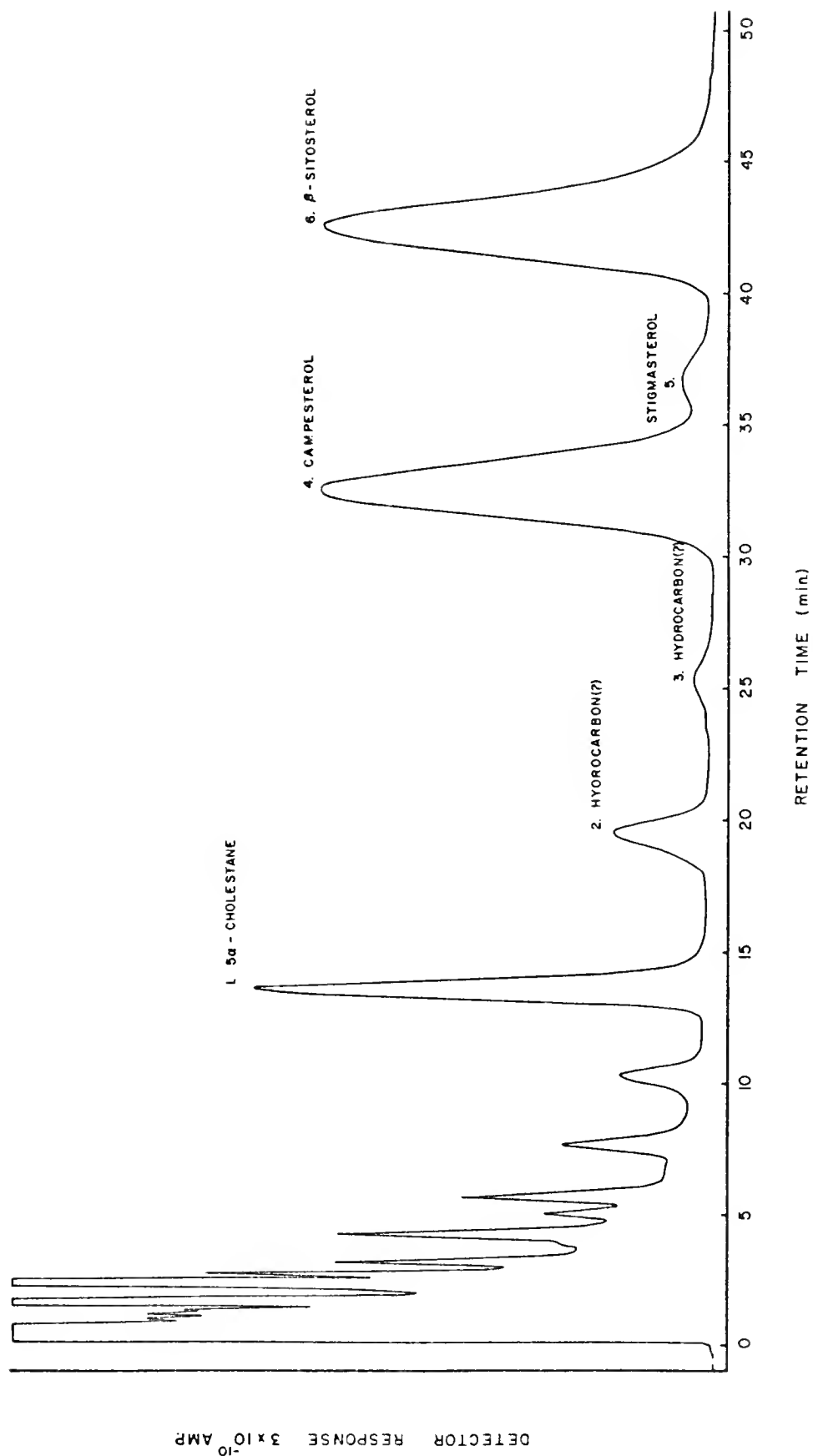
Figure 3. Gas-liquid chromatogram of sterols of 11-day-old longleaf pine seedlings.

Table 1. Relative retention, with respect to 5α-cholestane, of known sterols and those from 11-day-old long-leaf pine seedlings.

Component	Relative retention (r)[1]
Longleaf pine peak 2	1.44
Cholesterol	1.88
Longleaf pine peak 3	1.87
Campesterol	2.46
Longleaf pine peak 4	2.44
Stigmasterol	2.71
Longleaf pine peak 5	2.71
β-sitosterol	3.16
Longleaf pine peak 6	3.15

[1] Column characteristics: 5% OV-101 on Gas-Chrom Q (80-100 mesh), column temperature 250°C, carrier gas helium at a flow rate of 100 ml/minute; average retention time of 5α-cholestane was 13.6 minutes.

Table 2. Mass spectrum data of campesterol of germinating long-leaf pine embryos.

Fragmentation	Expected m/e	CAMPESTEROL Relative Intensity	
		Standard	Pine Sterol
Molecular ion [M$^+$]	400	95	93
M - 15	385	57	54
M - 18 (HOH)	382	95	96
M - [15 + 18]	367	64	65
M - [18 + 67(C_5H_7)]	315	58	58
M - [18 + 93(C_7H_9)]	289	93	98
M - [18 + 121(C_9H_{13})]	261	45	41
M - [18 + 108(C_8H_{12})]	274	31	18
M - side chain	273	42	42
M - [side chain + 18]	255	83	70
M - [side chain + 42]	231	52	51
M - [side chain + 18 + 42]	213	100	100
M - [side chain + 27]	246	18	13
M - [side chain + 27 + 17]	229	51	44

Table 3. Mass spectrum data of stigmasterol of germinating long-leaf pine embryos.

Fragmentation	Expected m/e	STIGMASTEROL Relative Intensity	
		Standard	Pine Sterol
Molecular ion [M$^+$]	412	53	48
M - 15	397	11	6
M - 18	394	26	29
M - [15 + 18]	379	12	10
M - [18 + 67]	327	6	12
M - [18 + 93]	301	18	15
M - [18 + 121]	273	23	25
M - [18 + 108]	286	5	12
M - side chain	273	23	25
M - [side chain + 18]	255	100	100
M - [side chain + 42]	231	18	27
M - [side chain + 42 + 18]	213	49	55
M - [side chain + 27]	246	5	8
M - [side chain + 27 + 17]	229	24	28

Table 4. Mass spectrum data of β-sitosterol of germinating long-leaf pine embryos.

| Fragmentation | β-Sitosterol | | |
| | Expected m/e | Relative Intensity | |
		Standard	Pine Sterol
Molecular ion [M$^+$]	414	97	97
M - 15	399	55	54
M - 18	396	93	90
M - [15 + 18]	381	59	62
M - [18 + 67]	329	54	52
M - [18 + 93]	303	93	92
M - [18 + 121]	275	39	36
M - [18 + 108]	288	15	13
M - side chain	273	42	42
M - [side chain + 42]	231	54	53
M - [side chain + 18]	255	79	76
M - [side chain + 42 + 18]	213	100	100
M - [side chain + 27]	246	21	16
M - [side chain + 27 + 17]	229	47	50

above the 299 m/e value. All the values from 299 to 420 were smaller than 1% of relative intensity and were suspected to arise from the background. The most intense peaks were at m/e 297 (8% of relative intensity), 157 (21%), 111 (19%), 97 (55%), 85 (26%), 83 (89%), 71 (49%), 69 (64%), 57 (100%), and 55 (63%). Because the mass spectrum data did not show any m/e value specific for cholesterol [153], it was concluded that the GLC peak 3 was not cholesterol but was probably an unknown long chain hydrocarbon.

From the results obtained by GLC and verified by combination of GLC-MS, it was therefore concluded that germinating longleaf pine embryos contained two major sterols, campesterol and β-sitosterol, and one minor sterol, stigmasterol. The esterified, glycosidic, and free sterol fractions contained the three above mentioned sterols. Some total and esterified sterol fractions also contained one additional minor peak having a relative retention value of 4.03.

Changes in Sterol Content upon Germination

All the sterol values reported here were the corrected values based on the recovery of radioactive materials through the extraction and fractionation procedures. The average recovery of total sterols was 82%. The recoveries of steryl esters and free sterols through silica gel were 91% and 90%, respectively.

On a dry weight basis, the total sterol content increased during seedling development (Fig. 4). By fractionation, three different forms of sterols were found in longleaf pine seedlings: steryl esters, steryl glycosides, and free sterols (Figs. 5, 6, and 7). The total sterol content was always higher than the summation of free, esterified, and glycosidic sterols. These total sterol values not only
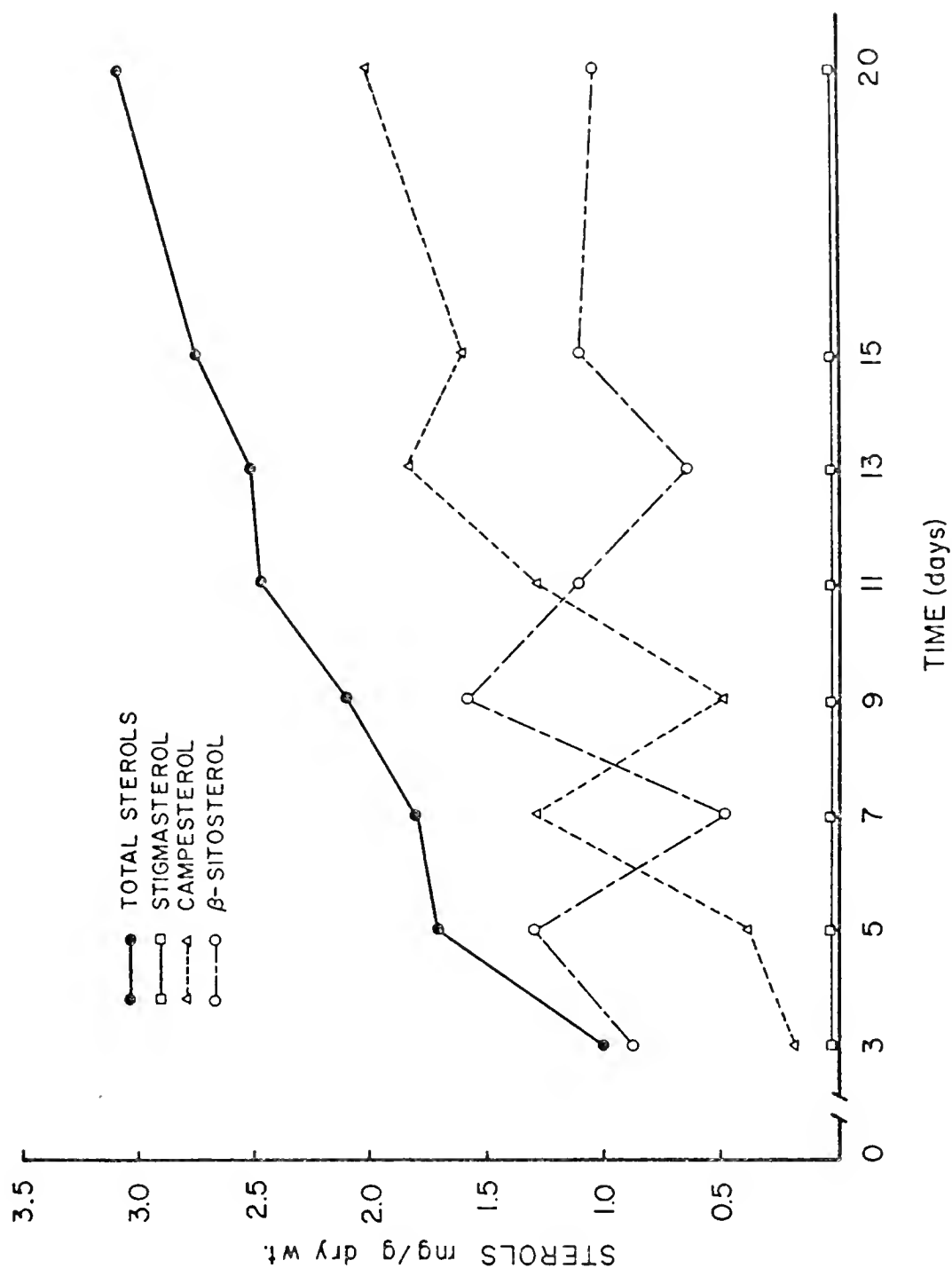
Figure 4. Changes in total sterol content and composition during germination of longleaf pine seeds.
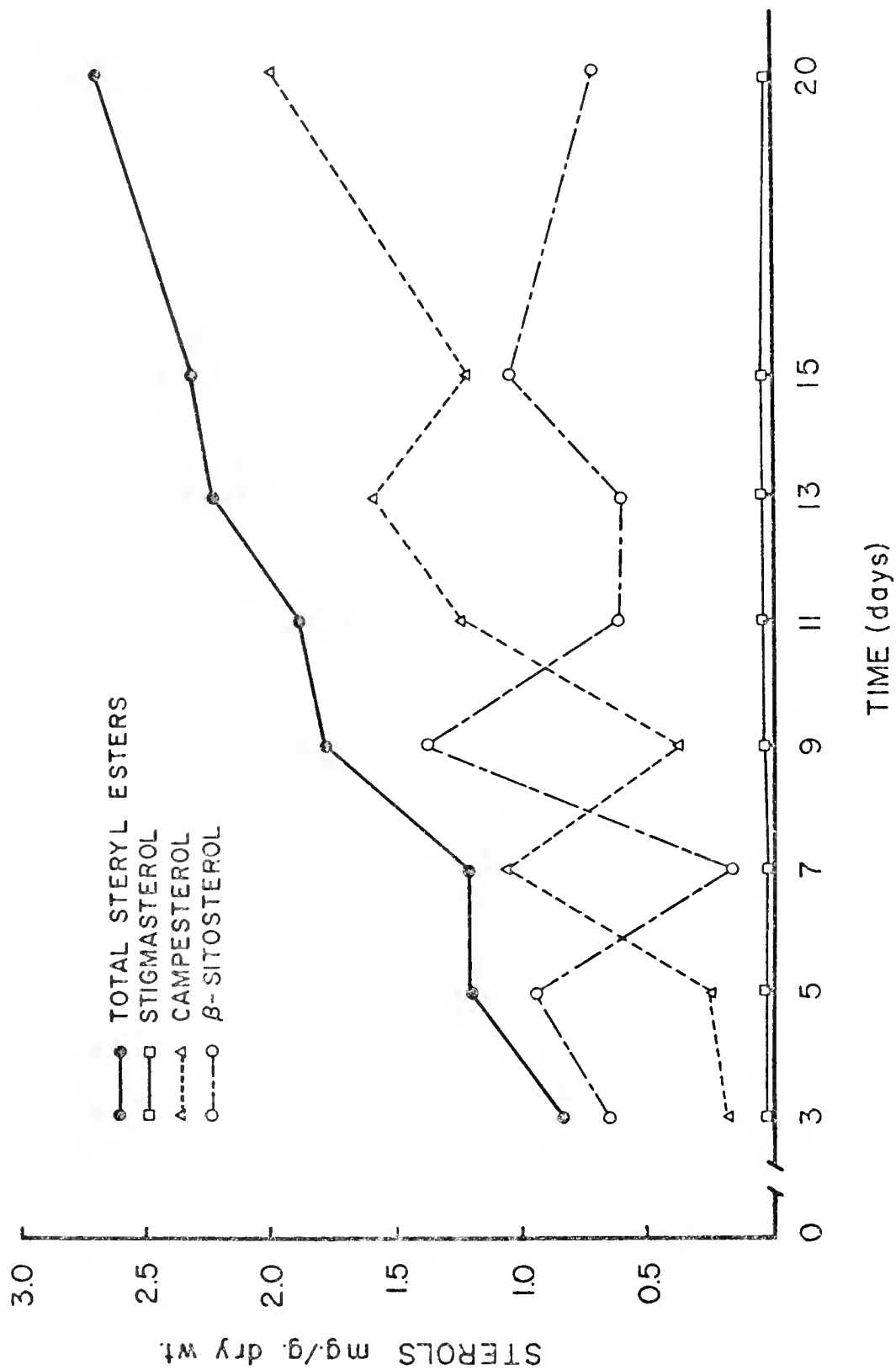
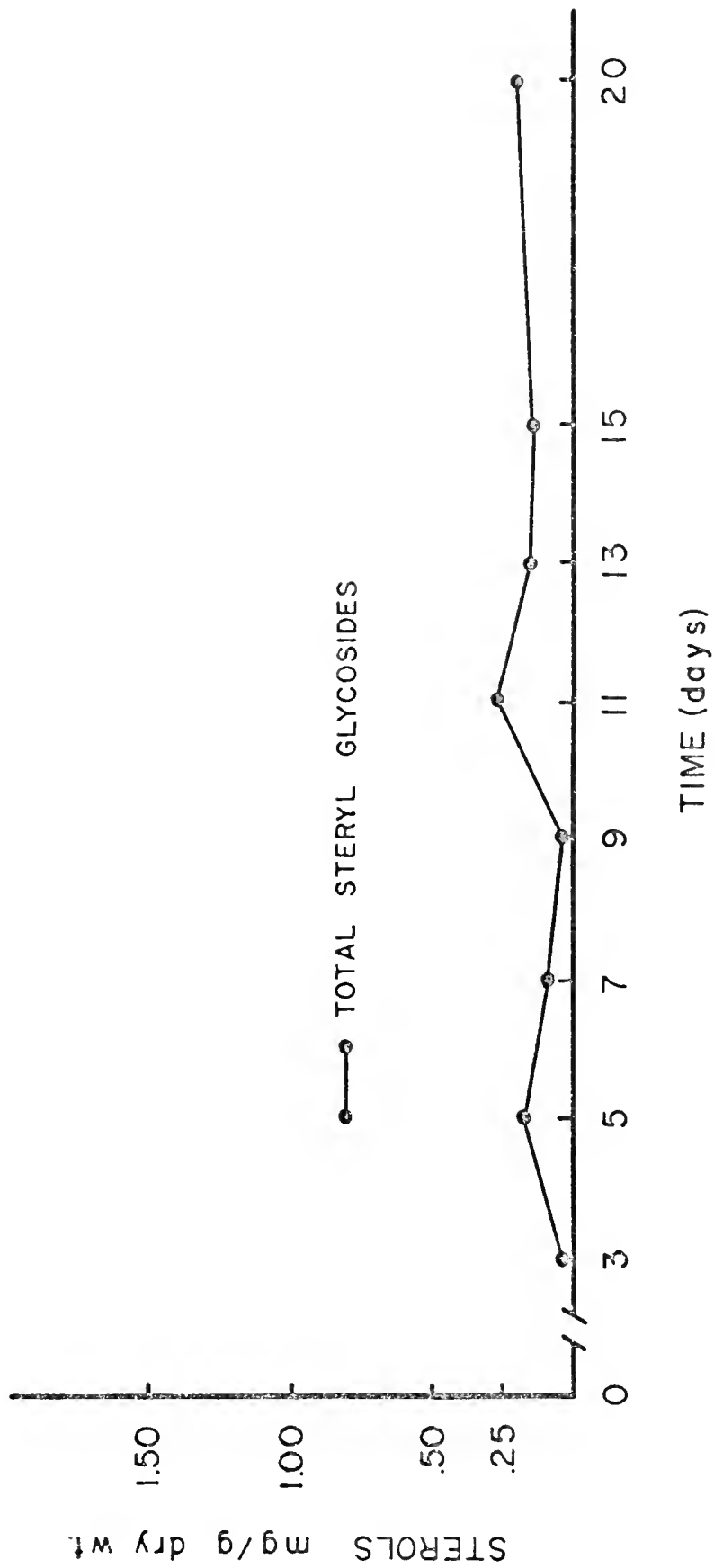Figure 5. Changes in steryl ester content and composition during germination of long-leaf pine seeds.

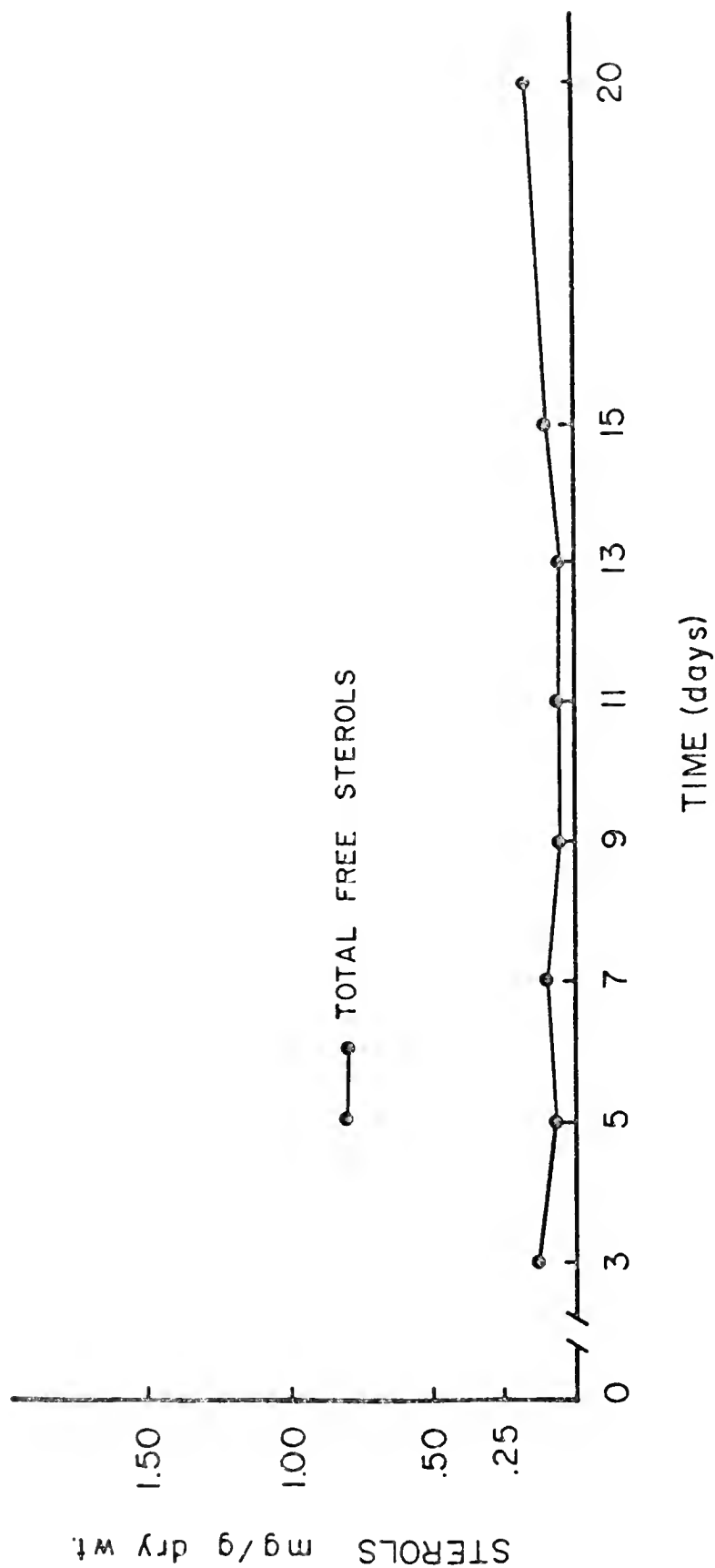Figure 6. Changes in steryl glycosides during germination of longleaf pine seeds.

Figure 7. Changes in free sterols during germination of longleaf pine seeds.

included the three forms mentioned above but also other sterol con-
jugates, such as the mono- and disulfates [47]. Fractionation also
revealed that the sterols were found mainly in the esterified fraction
which increased in amount during germination in much the same pattern
as the total sterol (Figs. 4 and 5). Steryl glycosides and free sterols
only made up a small portion of the total sterol content throughout
the germination period (Figs. 6 and 7).

In general, in the total and esterified sterol fraction, the
campesterol content increased with seedling development while the β-
sitosterol level remained approximately the same. The stigmasterol
content was very low and remained fairly constant throughout the whole
period of germination (Figs. 4 and 5). The data in Figs. 6 and 7
represented, respectively, the total glycosidic and total free sterol
content which occurred in very small amounts.

Effects of SKF 7997-A$_3$, ABA, and Cycloheximide on Total Sterol Biosynthesis

The germinating pine seeds were treated with SKF 7997-A$_3$, ABA,
and cycloheximide. In general, all the three inhibitors depressed
the biosynthesis of total sterol (Fig. 8). At 3 days, ABA slightly
increased the total sterol content over the control. At 7 and 12 days,
ABA depressed total sterol biosynthesis to 77% and 79% of the control,
respectively.

SKF 7997-A$_3$ had an effect on total sterol content during the whole
testing period. The sterol level was reduced to 50% of the control
at 3 days, 30% at 7 days, and 65% at 12 days.

Among the three inhibitors, cycloheximide had the greatest effect
on sterol biosynthesis. After cycloheximide treatment, the total
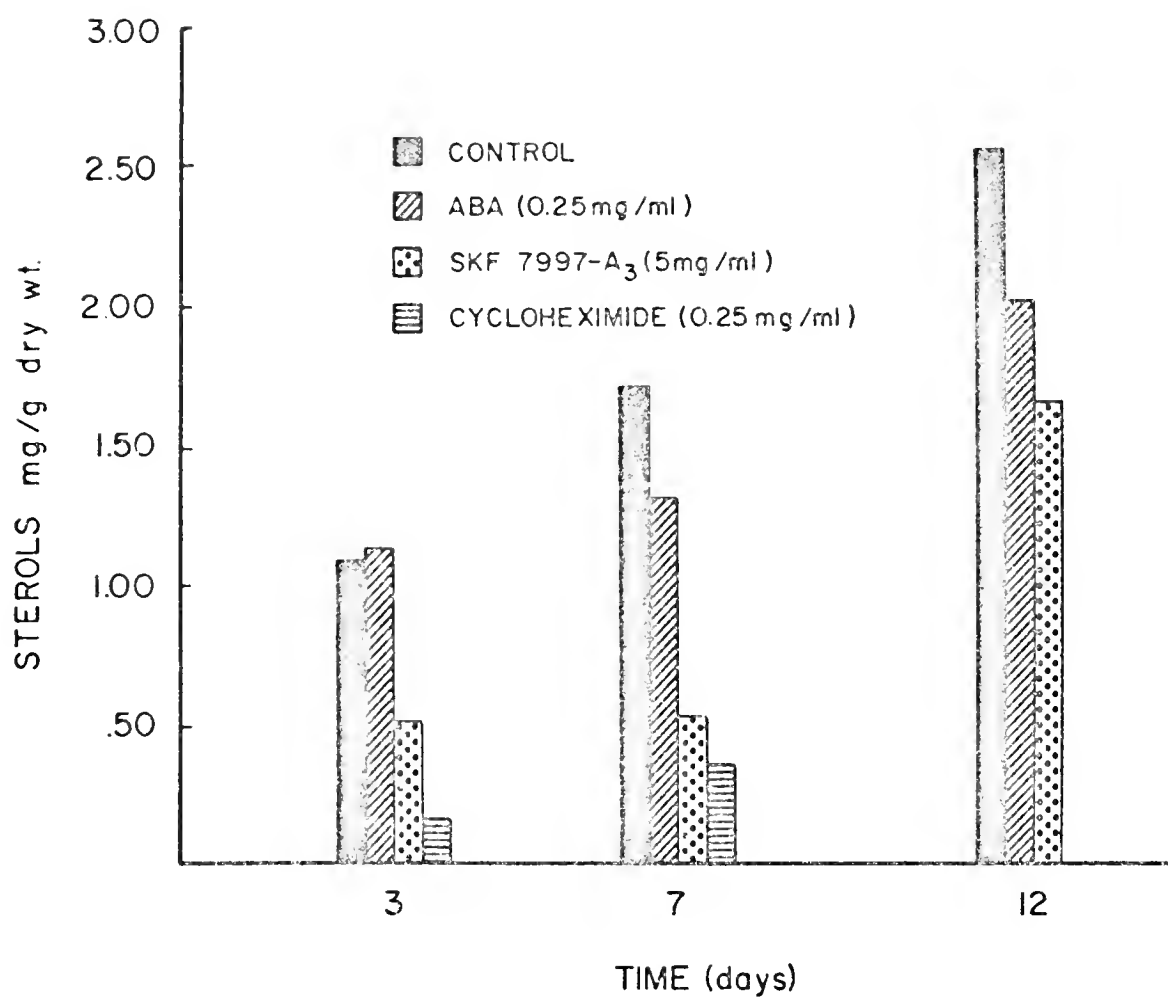
Figure 8. Effects of ABA, SKF 7997-A$_3$ and cycloheximide on total sterol content during germination of long-leaf pine seeds.
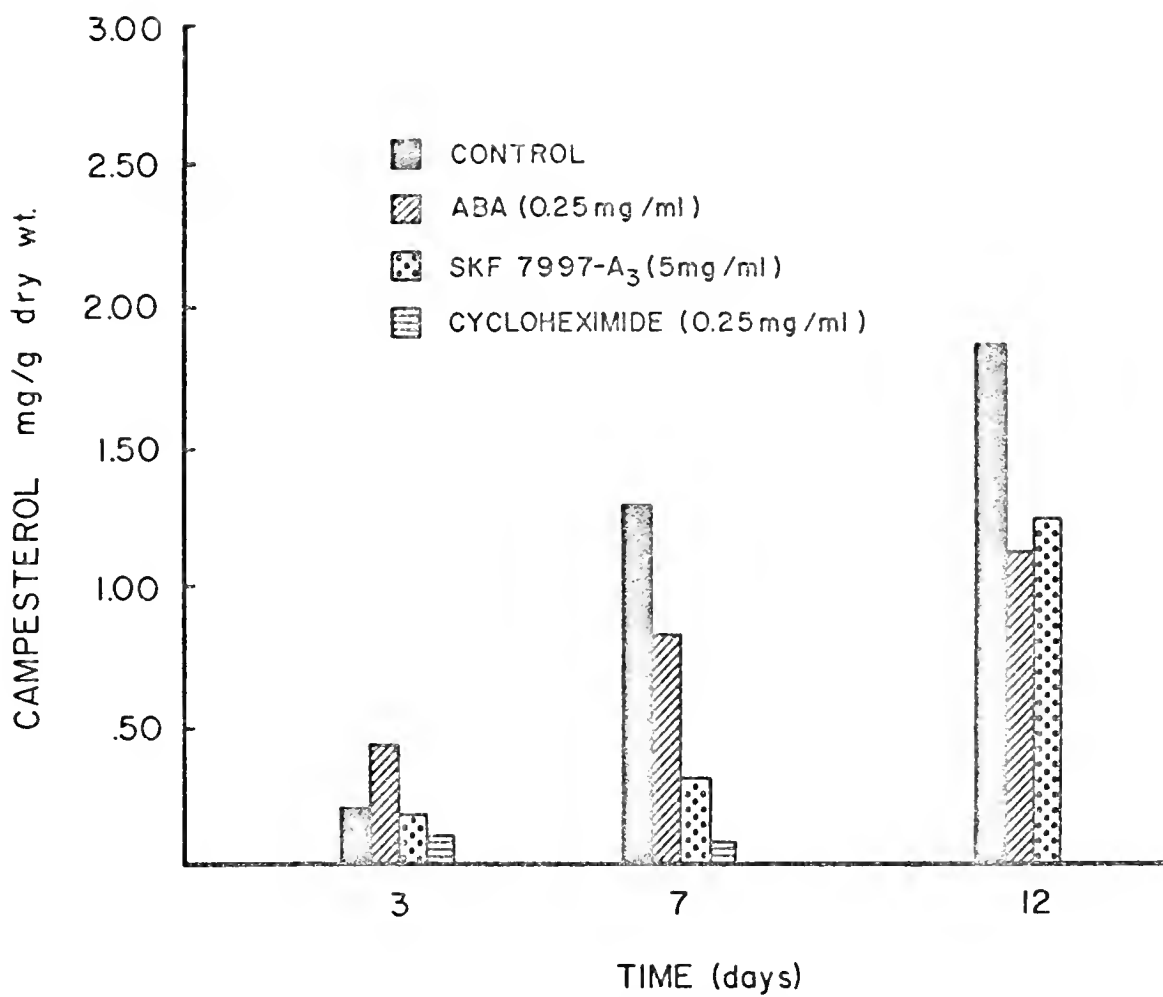
Figure 9. Effects of ABA, SKF 7997-A$_3$ and cycloheximide on the campesterol level during germination of long-leaf pine seeds.
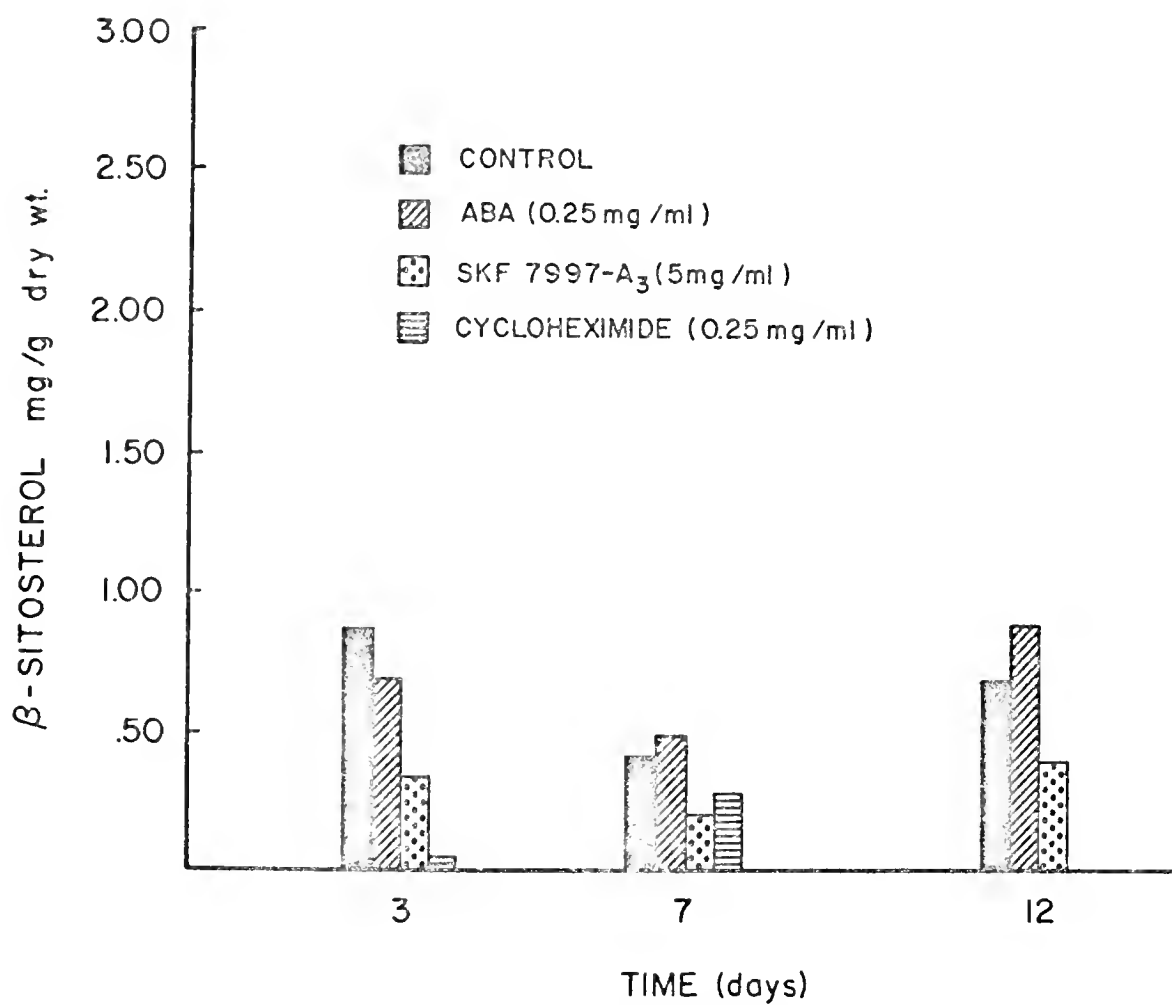
Figure 10. Effects of ABA, SKF 7997-A$_3$ and cycloheximide on the β-sitosterol level during germination of longleaf pine seeds.

sterol level was only 14% of the control after 3 days and 21% after 7 days. The germinating seeds treated with cycloheximide started to degenerate and rot after 9 to 10 days and were discarded.

ABA treatment increased the level of campesterol after 3 days but later inhibited and after 12 days, the campesterol level was only 60% of the control. ABA treatment, on the other hand, depressed β-sitosterol synthesis during the first 3 days, but increased it over the control at 7 days and 12 days (Figs. 9 and 10).

SKF 7997-A$_3$ caused a reduction of campesterol and β-sitosterol levels during the whole testing period. The greatest effect of SKF on campesterol was found at the 7-day stage when the campesterol level was only 24% of the control. After SKF treatment, the β-sitosterol level was only 38% of the control after 3 days and 58% of the control after 12 days.

Cycloheximide reduced the levels of both campesterol and β-sitosterol. The most pronounced effect was on β-sitosterol at the 3-day stage, with only 5% of the control, and on campesterol at the 7-day stage, with only 6% of the control.

Effects of the three Inhibitors on the Level of Reducing Sugars

All three inhibitors sharply reduced the level of reducing sugars throughout the test period (Fig. 11). The degree of inhibition at the 3-day stage was 24% (ABA), 36% (SKF 7997-A$_3$), and 47% (cycloheximide). At the 7-day stage, the inhibitions were 54% (ABA), 69% (SKF), and 87% (cycloheximide). After 12 days, the inhibitions were 80% (ABA) and 83% (SKF).
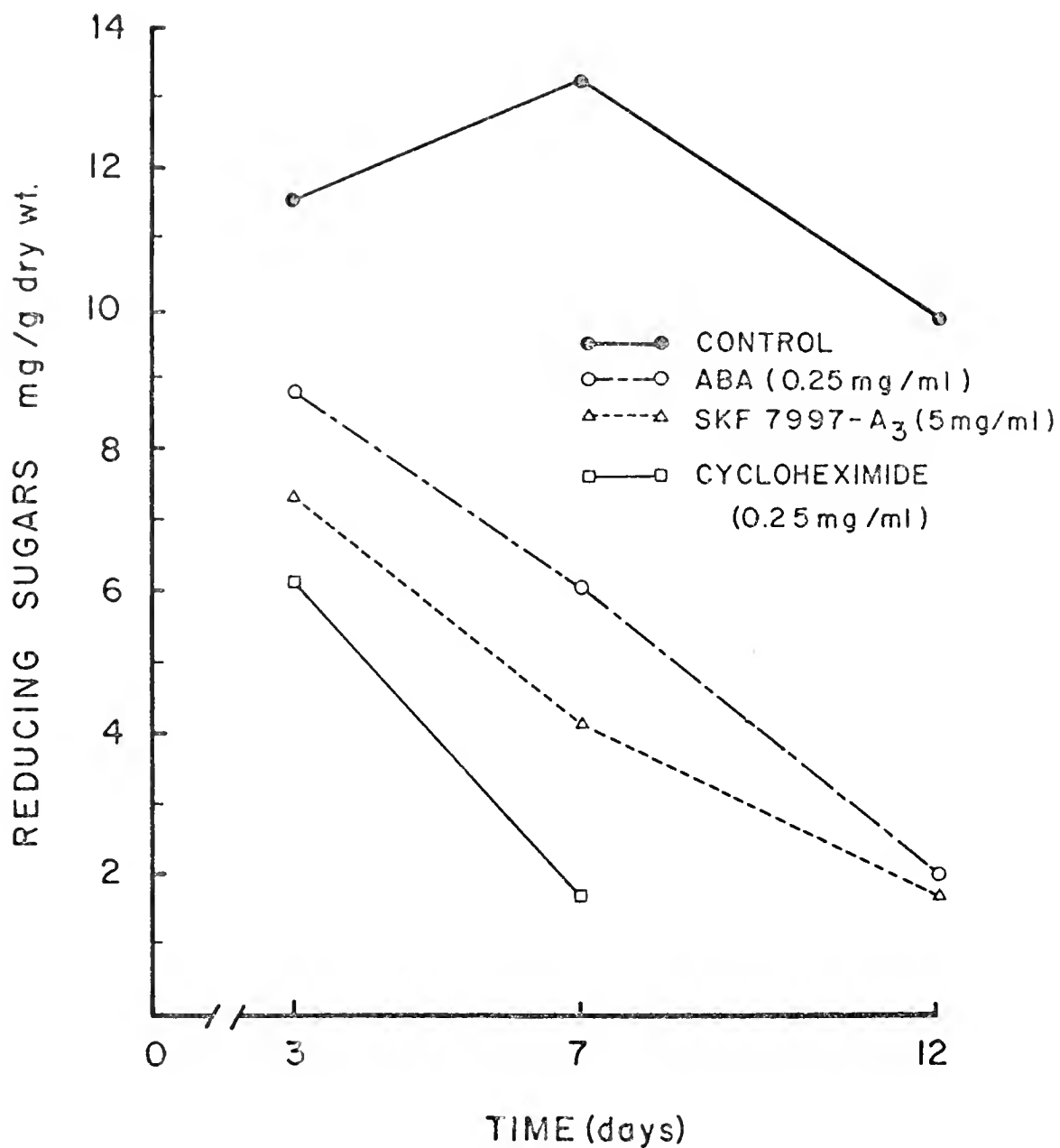
Figure 11. Effects of ABA, SKF 7997-A_3 and cycloheximide on the content of reducing sugars during germination of longleaf pine seeds.

Effects of the three Inhibitors on Total Chlorophyll
Biosynthesis

The effect of ABA on chlorophyll biosynthesis was less than that

of SKF 7997-A_3 and cycloheximide (Fig. 12). Both ABA and SKF depressed

the total chlorophyll content to the same level of 94% of the control

on the 3rd day, but at day 12, the SKF-treated seedlings contained

only 76% as much chlorophyll as the control, whereas ABA-treated

seedlings contained 89% as much as the control.

Cycloheximide-treated seedlings contained approximately 48% as

much chlorophyll as the control both at 3-day and 7-day stage.

Effects of SKF 7997-A_3 on the Activity of Isocitrate
Lyase

The participation of one of the key enzymes of the glyoxylate

cycle, isocitrate lyase, and its relation to germination of longleaf

pine seeds is presented in Fig. 13. When imbibed seeds were initially

removed from the seed coats, no activity of the enzyme was detected

in megagametophytes. The specific activity, however, increased

greatly by day 2 when protrusion of the radicle occurred, reached the

maximal level at day 8, remained unchanged for two additional days,

and then greatly declined by day 12. When the germinating embryos

were removed from the gametophytes on the 2nd day, the specific acti-

vity of the enzyme increased to the same level as the control at the

4th day, then the activity declined. But when the embryos were

removed from the gametophytic tissues immediately after imbibition at

0 day, isocitrate lyase was almost nil at the 2-day period, and then

increased and reached a maximal level at   day 4 which was only 34% of

the control.

When the decoated seeds were treated at 0 day with the steroid
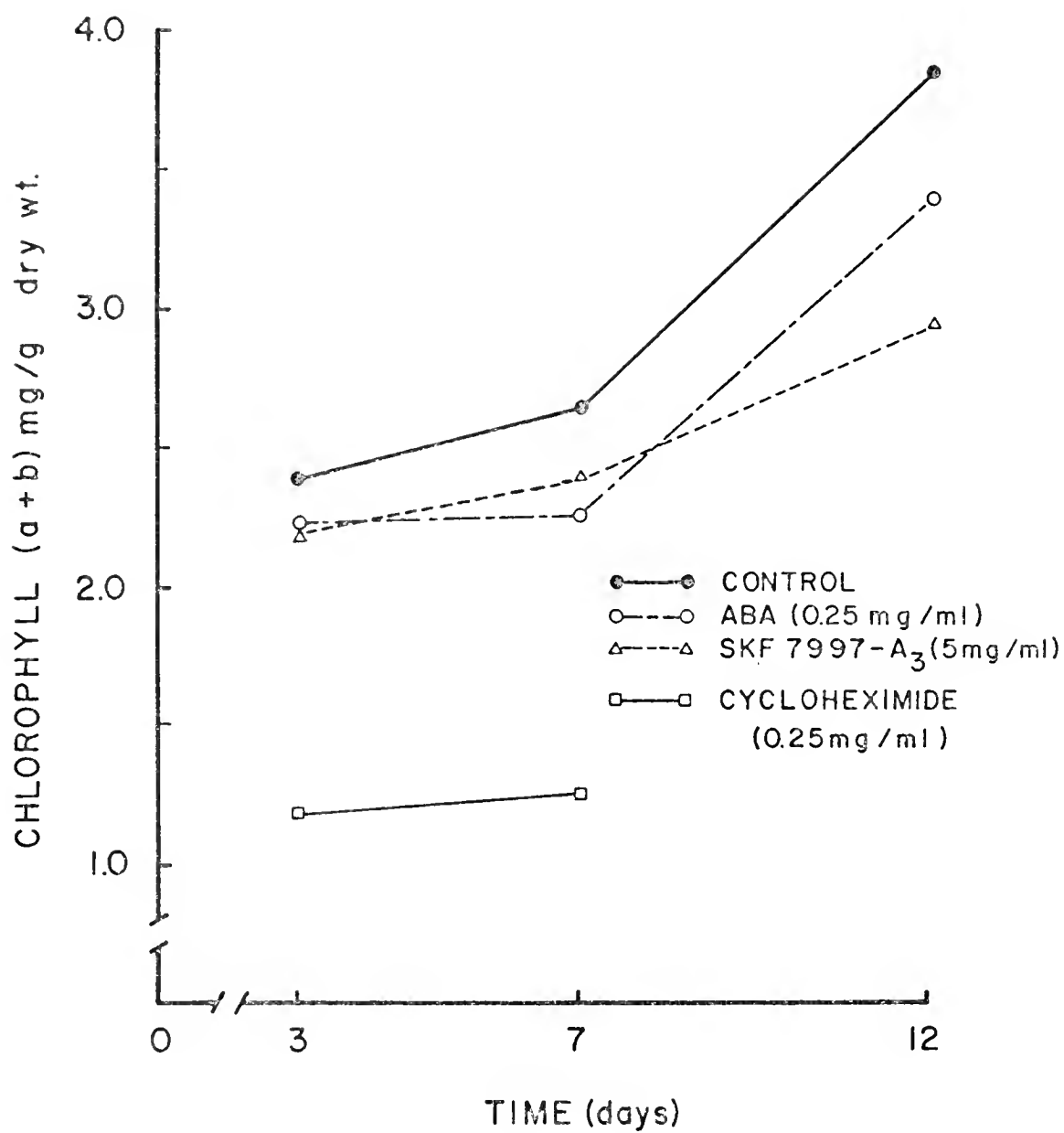
Figure 12. Effects of ABA, SKF 7997-A$_3$ and cycloheximide
on the chlorophyll content during germination
of longleaf pine seeds.
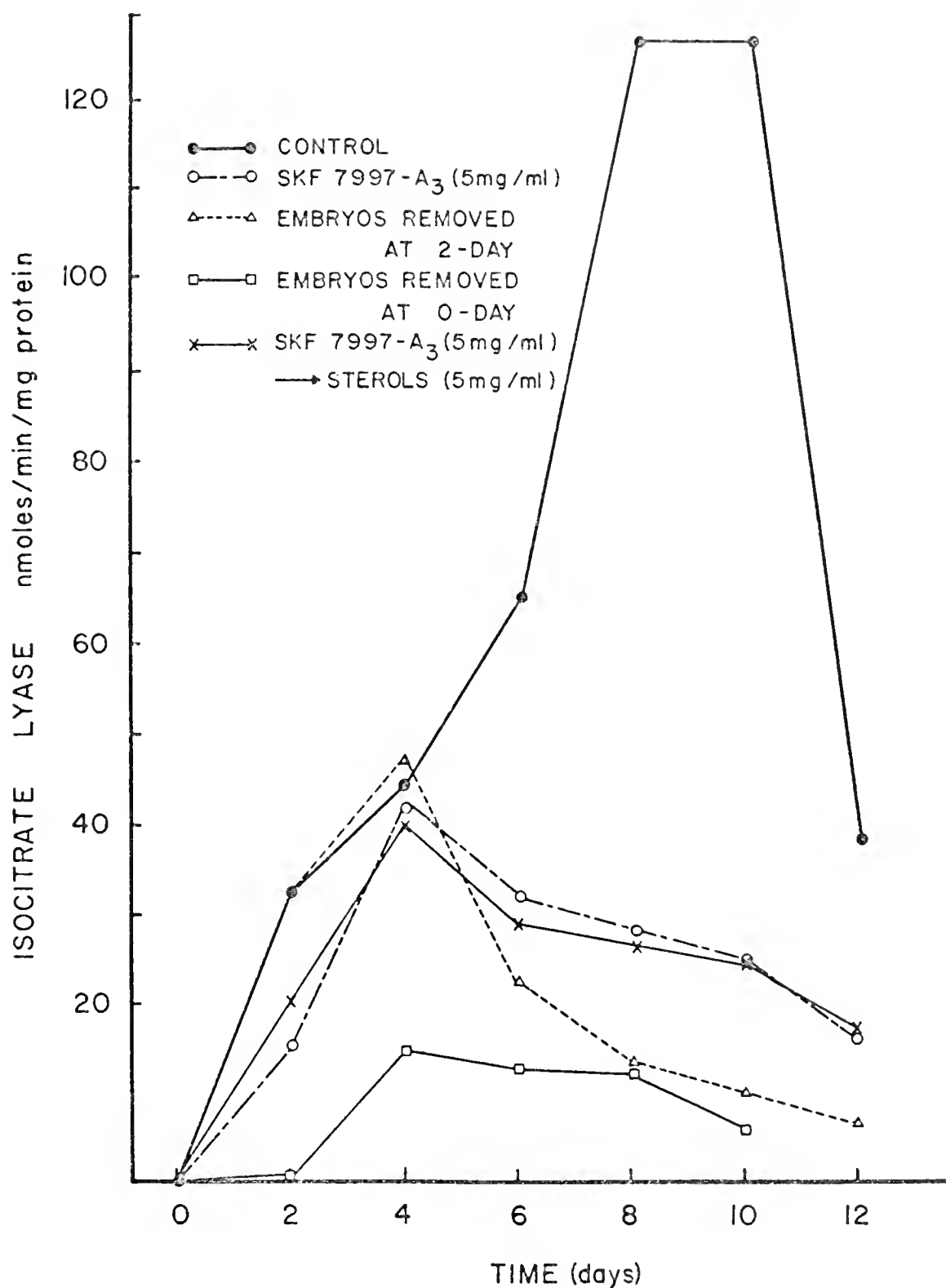
Figure 13. Changes of specific activity of isocitrate lyase in megagametophytes of germinating longleaf pine seeds.

inhibitor SKF 7997-A_3, the activity of the enzyme at day 2 was only about 50% of the control, but at day 4 was nearly the same as the control; the activity then declined slowly thereafter. After treatment of the SKF-treated seeds with a suspension of campesterol and β-sitosterol, the pattern of development of the enzymatic activity was approximately the same as for the SKF-treated seeds. No recovery of the activity of the enzyme was observed.

DISCUSSION

With the use of gas chromatography (GLC) and a combination of gas chromatography-mass spectrometry (GLC-MS), three sterols were isolated and identified in germinating longleaf pine embryos. Campesterol and β-sitosterol were the two major sterols, accounting for most of the sterol fraction, while stigmasterol only made up a very small portion of the total sterols. No trace of cholesterol was detected by GLC-MS, although there was one peak corresponding fairly closely to the retention time of authentic standard cholesterol.

By fractionation, three different forms of sterols, namely, steryl esters, steryl glycosides, and free sterols, were demonstrated to make up the total sterol content in germinating longleaf pine embryos. Steryl esters were the main sterol form, while steryl glycosides and free sterols were only minor fractions.

The total sterol content increased with time of germination, from 1.0 mg/g dry weight at 3 days to 3.1 mg/g dry weight at 20 days. The pattern of change in steryl esters was parallel to that in total sterols and accounted for most of the increase in total sterol content, while free sterols and steryl glycosides were low and remained fairly unchanged throughout the experimental period.

Changes in sterol content with time of germination have been studied in many different species of higher plants, and plant sterols were reported to occur mainly as free, esterified, and glycosidic sterols [44, 69, 111, 130, 145]. Sterol analyses during germination and seedling development in most cases showed that the total sterol

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content increased [44, 68, 70, 71, 72, 136]. These increases in sterol content suggest that sterols by themselves could play some role during growth and development of longleaf pine seedlings and in higher plants, in general. The definite roles of sterols and of different sterol forms in plants have not been elucidated, and many suggestions of function have been made in relation to the presence of different sterol forms in plants [44, 111, 130, 145]. Correlation analyses of different sterol forms by fractionation have been made as related to development and differentiation processes in order to find some clues of their physiological significance in plants. Experimental data obtained by different workers for different species are somewhat conflicting and make it difficult to answer some fundamental questions such as intracellular distribution of sterol forms, their function and their evolution in developing tissues [130, 145, 223].

In many reported cases, free sterols accounted for a large portion of the increase in total sterol content during germination [44, 73, 145], leading to the suggestion that free sterols are integral parts of plant cell membranes and that an increase in free sterol content during seedling development is a consequence of increased organelle genesis and new membrane synthesis [44, 111]. This hypothesis was mainly based on the observation by Grunwald that cholesterol in its free form was most effective in influencing the permeability of beet root and barley root cells as measured by the rate of leakage of β-cyanin and electrolytes, respectively [105, 109]. Stigmasterol, β-sitosterol, cholesteryl esters and cholesteryl glycosides were virtually ineffective in modifying leakages. Also, cholesterol was much more readily incorporated into erythrocyte membranes than the other plant sterols, due to its flat molecular configuration and to its side chain flexibility [77].

Cholesterol is a minor sterol in many plant species and is sometimes found only in traces [47, 62, 63, 69, 154, 181]. The most prominent higher plant sterols are β-sitosterol, stigmasterol, and campesterol [44, 47, 62, 63, 133]. The presence of cholesterol in higher plants was first reported in 1963 by Johnson et al. [138] who used thin-layer and gas-liquid chromatography. Since then, cholesterol was shown to occur in a number of plant species [44, 47, 62, 63, 69, 181]. The gas-liquid chromatograms of sterol fractions, in most cases, showed a minor peak appearing before that of campesterol and having a retention time corresponding closely to authentic standard cholesterol. This minor component, supposed to be cholesterol, accounted for less than 1%, or a trace, of the total sterol composition [47, 62, 63, 69]. Attempts to trap this minor GLC fraction for further analysis with mass spectrometry were not successful because the fraction was either absent or below the level of detection by mass analysis [47]. Thus, the reports on identity of cholesterol in tissues of Hordeum vulgare, Triticum aestivum, and Zea mays were tentative, based on the retention time of a component on the gas-liquid chromatograms [47, 62, 63]. Investigations on cholesterol in germinating seeds of Arachis hypogea, Phaseolus vulgaris, Pisum sativum, and Secale cereale also failed to demonstrate the presence of cholesterol [69].

Longleaf pine seedlings contain very little, if any, cholesterol, with the major sterols being β-sitosterol and campesterol, in addition to a small portion of stigmasterol. In conifers, β-sitosterol was identified from extracts of pollen from Pinus sylvestris and Pinus mugo [21], and both β-sitosterol and campesterol are present in the seeds of P. pinea [226] and P. elliottii [166] and in the barks of P. banksiana, P. contorta, P. lambertiana and P. taeda [203]. The major

sterol in pine stems was β-sitosterol, which accounted for 60-70% of the sterol composition and occurred as the free form, as esters of various fatty and aromatic acids, and as glycosides. No trace of stigmasterol was detected, and the gas chromatograms of samples from P. taeda and P. banksiana showed the presence of a very minor peak with the retention time of cholesterol [203]. A minor component was also identified in seeds and seedlings of P. elliottii [166].

The experiment on incorporation of cholesterol was not conducted using plant cell membranes, but using erythrocyte membrane in which cholesterol alone comprises approximately 25% of the total lipid membrane composition [162]. It is therefore not hard to understand why cholesterol is readily absorbed into erythrocyte membrane and not sitosterol, campesterol or stigmasterol which are completely absent in this animal membrane.

Young seedlings contain not only free sterols but also steryl esters and steryl glycosides [111]. Esterified sterols accounted for 83% of the total sterol content in tobacco leaves [107]. Cellular fractionation studies using these tobacco leaves also demonstrated that the steryl ester content was highest in the 20,000-46,000 g pellet, suggesting that most of the sterols in the tobacco leaves were associated with membrane-containing organelles and occurred in the esterified forms. In scutellum of maize, a rapid increase in steryl esters during germination was observed [145]. Also in Phaseolus vulgaris and Raphanus sativus, steryl ester content on a dry weight basis increased, although to a lesser extent than free sterols [69].

Bush [44], working on sterol changes during germination of tobacco seeds, found that the total sterols increased. This increase paralleled the increase in free sterols. The steryl esters also increased,

but to a lesser extent, while steryl glycoside content was low and decreased with time of germination. During germination of barley seeds, a marked increase in free sterols, esterified sterols, and esterified steryl glycosides was observed [130]. It was proposed that esterified steryl glycosides, with both polar and nonpolar regions of the molecule, were ideally suited as membrane constituents [130]. In the present study, the longleaf pine sterols were found mainly in the esterified forms which increased during germination in a way similar to the total sterol content. Similar patterns of increase in sterol content in different plant species were also reported [69, 70, 71, 72, 136]. Based on these observations, it could be that sterols may have a structural role, possibly as an integral part of the lipid layer of plant cell membranes [107, 111].

The evolution and localization of sterols in the needles of Pinus maritima was studied by David et al. in 1962 [61]. A gradual accumulation of sterols and fatty acids as lipid droplets during development of pine needles was registered during their first two years. There was a great increase in total sterol content from 0.3 mg/g fresh weight at the beginning of needle formation in the first year to 2.16 mg/g fresh weight at the end of the second year. This increase in sterol content paralleled an increase in fatty acids, showing the possibility that sterols may accumulate in droplets either in free or esterified forms [61]. In animal systems, cholesteryl esters were reported to constitute a large portion of the intracellular lipid droplets and serve as intermediates in the biosynthesis of steroids in the adrenal cortex of rats [24, 25, 60, 170, 179]. By analogy, it was also proposed in tobacco seedlings that stigmasterol and cholesterol were synthesized as, or rapidly converted to, esterified sterols

and then became associated with the lipid droplets of the cell where they may serve as a reserve pool for further steroidogenesis [45]. In germinating barley embryos, the free sterol content was rather high; however, the most striking observation was the increase in steryl esters within 5 days of germination [130]. The function of steryl esters in barley embryos was suggested to be involved in the transport of sterols and fatty acids released from triglycerides to the growing areas of the seedling for further synthesis and for membrane formation [130]. In plants, no evidence is available on the possible transport of sterols and fatty acids either in separate or combined forms from one site to another (e.g. from the reserve tissues to the germinating embryos). There is a report on intracellular sterol transport prob- ably from their site of synthesis to other organelles [145]. In long- leaf pine seeds, there are some interesting relationships between the high level of fats in megagametophytic tissues and large amounts of steryl esters present during the germination period. All of these facts, including the observation of lipid droplets in <u>Pinus</u> <u>maritima</u> needles, may provide a partial answer as to the possible function of steryl esters in growing pine tissues, in particular, and in higher plants, in general.

In addition to the possible structural role of sterols in plant cell membranes, the almost universal occurrence of these components in pines and other plants suggests that they might have some other definite functions. As yet, the nature of these possible functions remains quite obscure. Sterols are present in mitochondria of higher plants [74, 152], but no information is available on their possible function, other than structural, in these organelles. However, in

yeast, sterols are required for mitochondrial function [117]. Inhibition of sterol synthesis in yeast reduces respiratory competency which can be restored by addition of ergosterol [189].

Other effects of sterols on the metabolism of germinating seeds have been suggested: Sterols may operate at the level of transport of essential materials, such as a possible association of sterols with the transport of saccharides across the membrane [154]. Sterols may have an effect on the development of chloroplasts and photosynthesis [154]. Sterols may interact with proteins, such as enzymes, to control their functions [154].

Attempts to find further evidence of such effects in germinating longleaf pine seeds were made by using the animal steroid inhibitor SKF 7997-A_3, abscisic acid (ABA) and cycloheximide to determine their effects on the synthesis of sterols, chlorophyll content, and tissue concentrations of reducing sugars.

The average total chlorophyll content in green leaves of Pinus spp. is approximately 2.6 mg/g dry weight [163]. During germination of Pinus banksiana seeds, an increase in total chlorophyll content with time of germination was reported; at 9 days, the chlorophyll content was 0.9 µg/embryo [76].

Changes in reducing sugars in longleaf pine embryos occurred in a manner similar to that reported in Pinus luchuensis, Pinus taiwanensis and Pinus morrisonicola [217] and in the hardwood Acacia confusa [140] seeds during germination. In these three pine species, the level of reducing sugars increased, reached the maximum at 8-10 days, and then decreased thereafter [217].

SKF 7997-A_3 was reported to inhibit growth in peas [178] and to suppress floral induction in two short-day plants, Xanthium pensylvanicum

and Pharbitis nil [41], and in a long-day plant, Lolium temulentum [89]. In vitro, this animal steroid inhibitor inhibits the conversion of lanosterol to cholesterol, but in Xanthium leaves, although two principal compounds whose production was inhibited by SKF 7997-A$_3$ were identified as β-sitosterol and stigmasterol, the block is probably at more than one site and remains to be elucidated [41]. As has been shown experimentally, the biosynthesis of β-sitosterol and campesterol was depressed significantly in SKF 7997-treated pine seedlings. A change in sterol content in the membrane could strongly affect the transport of metabolites across the membrane. The results obtained showed that SKF 7997-A$_3$ sharply depressed the level of reducing sugars and significantly reduced the total chlorophyll content.

Abscisic acid (ABA) also inhibited the sterol content in long-leaf pine, but to a lesser degree than SKF 7997-A$_3$ and cycloheximide. Campesterol was decreased with a slight increase in β-sitosterol. The ability of ABA to induce dormancy in seedlings of a number of species has been reported [93, 111]. In longleaf pine seedlings, decreased levels of campesterol and increased levels of β-sitosterol with ABA treatment had similar patterns at the 3-day and 5-day germination period. No information is available on the possible relationship of the individual sterols such as β-sitosterol to seed dormancy; however, β-sitosterol in dry mature Pisum sativum seeds was reported to be the predominant sterol in all fractions [177] and was present to the extent of 0.67 mg/g of dried seed [12, 13]. In potato tubers (Solanum tuberosum) during storage, β-sitosterol was the predominant sterol [75]. The relatively high level of β-sitosterol in these situations may indicate the initiation of dormancy by ABA.

Abscisic acid (ABA) also depressed the synthesis of reducing sugars and chlorophyll in longleaf pine seedlings. Its effect was comparatively less than that caused by SKF 7997-A$_3$. The effects of ABA on the synthesis of sterols and chlorophyll were reported in corn shoots [176]. By measuring the incorporation of labeled mevalonic acid into the sterol fraction of ABA-treated and control corn shoots over a finite experimental period, it was shown that ABA had no inhibitory effect on sterol biosynthesis in corn. After incubating etiolated corn shoots in the light with and without ABA and subsequently comparing the levels of chlorophyll in the tissues, it was found that ABA depressed the synthesis of chlorophyll by 42% [176]. Also ABA greatly decreased the chlorophyll level of excised embryos of Fraxinus ornus [213]. In isolated leaf discs, ABA accelerated the loss of chlorophyll in all species examined [7, 83, 209].

ABA reduced the level of reducing sugars nearly as much as SKF 7997-A$_3$ did. The inhibition of ABA on α-amylase and ribonuclease was reported in barley aleurone layers [54, 56]. In the case of longleaf pine, ABA may inhibit the synthesis of certain specific enzymes required for formation of reducing sugars. ABA may exert its action in a manner similar to that in barley grains by inhibiting the synthesis of the enzyme-specific RNA molecules that are required for the expression of the gibberellic acid effect, or by preventing the incorporation of these RNA molecules into an active enzyme-synthesizing unit [56, 93].

The antibiotic cycloheximide is of considerable interest since it is a potent inhibitor of protein synthesis at the translational level [168]. In cycloheximide-treated longleaf pine seedlings, a strong suppression of sterols, reducing sugars and chlorophyll was observed.

Cycloheximide was reported in isolated barley aleurone layers to inhibit the production of α-amylase and ribonuclease to a greater extent than did ABA [55, 56]. ABA inhibited the synthesis of α-amylase after a lag of 2 to 3 hours while the addition of cycloheximide (10 μg/ml) to barley aleurone layers resulted in an immediate cessation of α-amylase synthesis and also the incorporation of ^{14}C-leucine into the cellular proteins of aleurone layers [56].

The suppression of the level of reducing sugars by SKF 7997-A$_3$ suggests that sterols may have an influence on some particular enzyme in steps transforming fatty acids to sugars. Pine seeds contain an embryo deeply embedded in gametophytic tissues which serve as a lipid reservoir supporting the early growth of the young seedling [53]. However, lipids are not transported directly from megagametophyte to the embryo and must be transformed into sugars via the glyoxylate cycle [53]. Isocitrate lyase, a key enzyme of the glyoxylate cycle, which is almost nil in ungerminated seeds, increases in activity many-fold during germination, accompanied by a decrease in lipid content of the gametophyte. Once lipids are exhausted, the enzyme activity is markedly reduced [37, 53, 91, 92].

The participation of isocitrate lyase in converting lipolytic products of acetyl-CoA to carbohydrate in germinating megagametophytes of longleaf pine seeds was assayed. No isocitrate lyase activity was found in the ungerminated seeds. During the first few days of germination, the activity of the enzyme increased dramatically with a peak at 8 days, approximately the time lipid breakdown and carbohydrate synthesis was most rapid. Later on, as the lipid content declined in the later stages of germination, so did the activity of the enzyme. Similar patterns of enzymatic activity development was observed in

the germinating seeds of Pinus ponderosa [37, 53], Pinus pinea [92], and in other fatty germinating seeds such as Citrullus vulgaris [128] and Cucurbita pepo [50].

When longleaf pine seeds were treated with SKF 7997-A$_3$, there was a sharp suppression of enzyme activity after the fourth day of germination. It is suggested therefore that sterols may have an effect on the regulation and developmental activity of this enzyme. No information is available on the possible interaction of sterols and enzymes occurring in plant systems. It is reported that there is the formation of a complex between the enzyme trypsin and ergosterol, yielding a more stable and more reactive enzyme towards egg albumin than the uncomplexed enzyme [188]. The lack of recovery of enzyme activity after treatment with sterols may be due to the insolubility of sterols, the probability of SKF 7997 to be a noncompetitive inhibitor, or to produce irreversible inhibition of the enzyme through chemical modification of its structure, as the action of diisopropyl-phosphofluoridate (DFP) on acetylcholineesterase and other enzymes possessing an essential reactive serine residue at their active sites [168], or the possibility of SKF 7997-A$_3$ changing the expression of genetic information stored in the chromosomes that cannot be restored by addition of sterols.

Further attempts were made by removal of the embryos from the megagametophytes at 0-day and at 2-day and following the change in isocitrate lyase activity as related to the embryonic induction factors. This experiment was based fundamentally on the observations by Bilderback in Pinus ponderosa [37] and by Young and Varner in Pisum sativum [229, 238]. In ponderosa pine, the isocitrate activity continued to develop normally when the embryo was removed two days, but

not immediately, after stratification [37]. The same result was obtain-
ed with phosphatase in peas [229, 238]. It has been suggested that
events occurring in the reserve tissues are under embryonic control,
and that the embryo could play an active regulatory role by producing
some substance resulting in enhanced enzyme production in pine megagame-
tophytes [37]. In ponderosa pine seeds, this unknown embryonic factor
cannot be replaced by gibberellic acid (GA_3), indoleacetic acid (IAA),
and benzyladenine (BA); however, when isolated gametophytes were
incubated with the embryo diffusate, a substantial increase in activity
of isocitrate lyase nearly to the control was observed [37]. Observa-
tions of limited increase of enzyme activity in longleaf pine gameto-
phytic tissues when embryos were removed 2 days after germination did
not permit further work on the theoretical embryo substance, supposed
to be a sterol. Also the insolubility of sterols in aqueous solution
did not allow testing of the possible interaction of sterols with the
enzyme isocitrate lyase.

SUMMARY

Very little work has been done on sterols in germinating coniferous seeds. The object of this study was to follow the occurrence and variations of different forms of sterols and to investigate their possible physiological functions during germination of longleaf pine seeds (Pinus palustris Mill.).

Pine seeds were germinated in a growth chamber under controlled conditions, and the germinating embryos or young seedlings were used for extraction and fractionation of sterols and their derivatives. By using gas-liquid chromatography (GLC) and combination of GLC-mass spectrometry (GLC-MS) for sterol identification and analysis, three different components, campesterol, stigmasterol and β-sitosterol, were detected in the pine sterol composition. Campesterol and β-sitosterol were two major sterols while stigmasterol was only present in small amounts throughout the whole experimental period. Fractionation revealed three different forms of sterols in pine tissues: steryl esters, steryl glycosides, and free sterols. Steryl esters were the main sterol fraction, while free sterols and steryl glycosides were only minor ones. All the three sterol components were present in these fractions.

The total sterol content increased with time of seedling development, from 1.0 mg/g dry weight at 3 days to 3.1 mg/g dry weight at 20 days. The changes in steryl esters paralleled total sterols, while steryl glycosides and free sterols were low and remained fairly constant throughout the germination period. The campesterol content increased with seedling development in both the total sterol content and the

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steryl ester fraction, while the levels of β-sitosterol and stigmasterol were nearly the same.

Cycloheximide, abscisic acid (ABA) and SKF 7997-A$_3$ significantly inhibited the biosynthesis of sterols, reducing sugars, and total chlorophyll in longleaf germinating pine seeds. SKF 7997 and cycloheximide reduced the levels of both β-sitosterol and campesterol, while the application of ABA caused a decrease in campesterol and a slight increase in β-sitosterol. A depression of reducing sugars and chlorophyll by SKF 7997 could indicate that there is a possible operation of sterols at the transport level of essential materials, such as saccharides across membranes, and that the formation and development of chloroplasts may be under the partial control of sterols.

SKF 7997 also sharply reduced the activity of the enzyme isocitrate lyase. The application of campesterol and β-sitosterol to the SKF 7997-treated seeds did not reverse the inhibition caused by the animal steroid inhibitor. The depression of isocitrate lyase activity by SKF 7997 indicates that this steroid inhibitor has an influence on a catalytic protein that may influence germination, but it will require further experimentation to determine if sterols have a direct regulatory role in pine seed germination.

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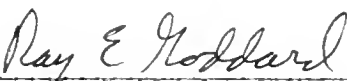
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BIOGRAPHICAL SKETCH

Cu Van Vu was born June 29, 1942 in Lam Dong, South Vietnam. In
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Physics, Chemistry and Biology in 1962 and was admitted to the School
of Medicine, University of Saigon. Because of health conditions, he
dropped out of the Medical School a short period after and was admitted
to the College of Agriculture in Saigon in November 1962. He continued
his studies at the same time at the College of Basic Sciences, University
of Saigon, and earned the certificate of Physics, Chemistry and Natural
Sciences in 1963. In November 1965, he received the degree of Engineer
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In August 1970 he was awarded a fellowship for going to the U.S. for
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been pursued at the University of Florida in forest tree physiology.
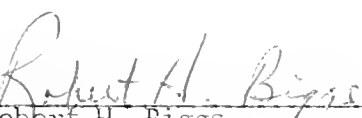
Cu Van Vu is a member of XI SIGMA PI and of the Society of
American Foresters. He is married to the former Tieng Tran and has a
two-year-old son, Thien.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Ray E. Goddard, Chairman
Associate Professor, Forestry

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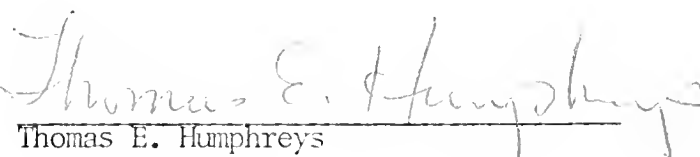
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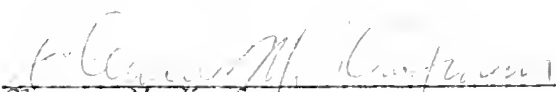
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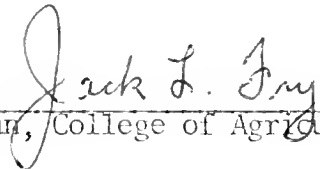
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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

March, 1976

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